MECHANISM(S) UNDERLYING THE ATTENUATED ACTH RESPONSES TO STRESS IN PREGNANCY

Shuaike Ma

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For my mother, father and my family

Declaration

The studies described in this thesis were undertaken by me in Laboratory of Neuroendocrinology, Division of Biomedical and Clinical Laboratory Sciences (DBCLS), University of Edinburgh, Medical School, under the supervision of Professor J A Russell and Dr. Mike Shipston. All of the work described was performed by me in collaboration with others as indicated below:

1) Surgery was performed by Prof. J A Russell, Drs Alison Douglas and Phillip Bull.
2) Dissection of the hypothalamus and the median eminence was performed by Prof. J A Russell.
3) CRH concentration was assayed by Prof. Pierluigi Navarra (Catholic University Medical School, Italy).

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Abstract

The aim of this project was to investigate the possible mechanism(s) underlying the attenuated hypothalamic-pituitary-adrenal (HPA) axis response to stressors during pregnancy at the level of the anterior pituitary. We found that the reduced HPA axis activity during pregnancy was not due to changes in the sensitivity of corticotrophs in the anterior pituitary, but to the decreased secretion particularly of vasopressin and possibly corticotropin-releasing hormone (CRH) from the hypothalamus. The reduced CRH and vasopressin secretion during pregnancy is a result of action of neurosteroid metabolites of progesterone as a 5α-reductase inhibitor reversed the attenuated adrenocorticotrophic hormone (ACTH) secretory response to stress.

By *in situ* hybridisation, we found a progressive decrease in proopiomelanocortin (POMC) mRNA expression in the anterior pituitary during pregnancy. However, there was no change in ACTH content. Compared with expression in the anterior pituitary of virgin rats, in late pregnancy (day 21), CRH receptor subtype 1 (CRHR1) mRNA expression was not changed, vasopressin receptor subtype 1b (V1bR) mRNA expression was reduced, and glucocorticoid receptor (GR) mRNA was increased. We also measured large conductance potassium channel (BK) and the splice variant of the BK channel, STREX, mRNA expression and found no change during pregnancy. Corticotropin-releasing hormone binding protein (CRHBP) mRNA expression was elevated by 30 min restraint stress in the anterior pituitary, but there were no differences between virgin and pregnant rats.

In vivo, a CRHR1 antagonist, antalarmin, decreased ACTH secretion in response to swimming stress in both virgin and pregnant rats. However, a V1a/b receptor antagonist decreased the ACTH response to this stressor in virgin rats only. Exogenous vasopressin or CRH injection alone increased ACTH secretion more in virgin than in pregnant rats. Injection of CRH and vasopressin together had a similar stimulatory action on ACTH secretion in virgin and pregnant rats. Thus the attenuated ACTH stress response during pregnancy is primarily a result of reduced vasopressin secretion during stress.

To investigate whether corticotroph sensitivity to CRH and vasopressin is modulated during pregnancy, we incubated anterior pituitary cells with different concentrations of CRH, vasopressin, a combination of CRH and vasopressin, or cAMP. We found a dose related augmentation of CRH stimulation of ACTH secretion by vasopressin. The sensitivity of corticotrophs to CRH was greater in pregnancy and the augmentation by vasopressin of the effects of a low concentration of CRH was greater for corticotrophs from pregnant than from virgin rats. A greater effect of cAMP suggests changes in post receptor signalling mechanisms in pregnancy.

GABAergic mechanisms inhibit the HPA axis via $GABA_A$ receptors on CRH neurones and their inputs. Allopregnanolone, the neurosteroid metabolite of progesterone, acts as a major $GABA_A$ receptor allosteric modifier, so enhancing the action of GABA. To investigate such a possible enhanced GABAergic action by allopregnanolone on CRH and vasopressin secretion during pregnancy, we prevented the inhibitory action of the metabolic product of progesterone by blocking production via 5α-reductase with 4-MA. 4-MA prevented the attenuated ACTH secretory response to swimming stress in pregnant rats and had no effect directly on the corticotrophs.

The reduced stress- induced ACTH response during pregnancy is predominantly the consequence of reduced vasopressin release, with secondary changes in corticotrophs. The central changes may be due to the inhibitory action of allopregnanolone on CRH and vasopressin secretion from the hypothalamus.

Contents

Contents

List of figures

List of tables

Abbreviations

11β-HSD	11β-OH steroid dehydrogenase
11β-HSD1	type 1 11β-OH steroid dehydrogenase
11β-HSD2	type 2 11β-OH steroid dehydrogenase
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
α-MSH	α-melanocyte-stimulating hormone
β-LPH	β-lipotropin
BK-channel	large Ca^{2+}-activated K^{+}-channel
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBG	corticosteroid-binding globulin
cDNA	complementary DNA
CNS	central nervous system
CRH	corticotropin-releasing hormone
CRHBP	CRH- binding protein
CRHR1	CRH type 1 receptor
CRHR2	CRH type 2 receptor
DAG	diacylglycerol
DOC	deoxycorticosterone
DTT	dithiothreitol
GABA	gamma-aminobutyric acid
GR	dlucocorticoid receptor
Gs	stimulatory guanine nucleotide-binding protein
hnRNA	heterogeneous nuclear RNA

HPA	hypothalamus-pituitary-adrenal
IP_3	inositol-1,4,5 trisphosphate
LC	locus coeruleus
ME	median eminence
MR	mineralocorticoid receptor
mRNA	messenger RNA
NADPP	nicotinamide adenine dinucleotide phosphate
NSB	non specific binding
phADX	pharmacological adrenalectomy
PI	phosphatidyl inositol
PKA	protein kinase A
PLC	phospholipase C
PMA	phorbol myristate acetate
POMC	proopiomelanocortin
PVN	paraventricular nucleus
SON	supraoptic nucleus
TBPS	*t*-butylbicylophosphorothionate
TC	total count
THDOC	allotetrahydrodeoxycorticosterone
V1a receptors	vasopressin receptor subtype 1a
V1b receptors	vasopressin receptor subtype 1b
V2 receptors.	vasopressin receptor subtype 2
UTP	uridine triphosphate
UTR	5'-untranslated region

Publications from this thesis

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Chapter 1

General introduction

1.1 Stress and the hypothalamus-pituitary-adrenal (HPA) system

Selye defined Stress as 'a non-specific response of the body to any demand made on it' (Selye 1975). However, this non-specific concept has been modified as the ways in which different stressors are processed have become understood. The stress response system consists of central and peripheral components (Figure 1.1). The hypothalamus-pituitary-adrenal axis (HPA) is the most prominent system mediating physiological adaptations to stress. Central components include: the corticotrophin-releasing hormone (CRH) and arginine-vasopressin parvocellular neurones of the paraventricular nuclei (PVN) of the hypothalamus; nuclei of the medulla including the catecholaminergic neurones of the locus coeruleus (LC) and other cells groups of the medulla and the pons. Peripheral components include the anterior pituitary-adrenal axis and the efferent sympathetic/adrenomedullary systems (Stratakis & Chrousos 1995). The endpoint of the stress response of the HPA system, an elevation of plasma corticosteroid (in rat and mouse, or cortisol in human) levels is usually considered to be definitive of a state of stress.

The activity of the HPA axis is regulated by a variety of stimulatory and inhibitory factors. The former are CRH, vasopressin, oxytocin and other stimulatory factors, the latter are glucocorticoids by negative feedback, brain inhibitory transmitters, local negative modulation such as CRH-binding protein and other inhibitory factors including atrial natriuretic peptide (Figure 1.1).

Figure 1.1 Diagrammatic summary of HPA axis response regulation

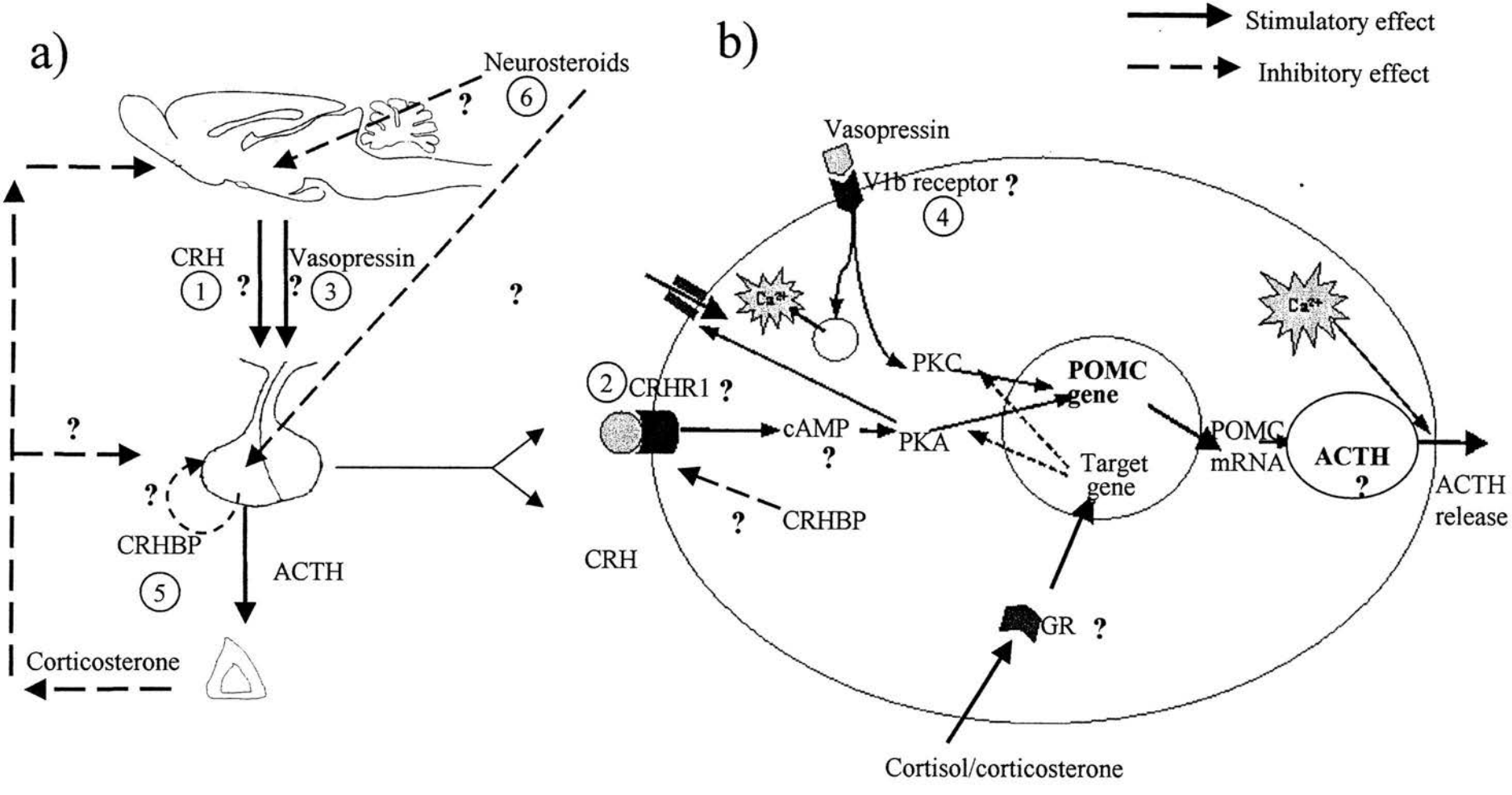


Figure 1.1 Diagrammatic summary of HPA axis response regulation a) factors regulating the system b) regulation of ACTH secretion from corticotrophs.ACTH= adrenocorticotrophic hormone; GR= glucocorticoid receptor; PKA= protein kinase A; PKC= protein kinase C.
① ② details in Chapter 1.3.1; ③ ④ details in Chapter 1.3.2; ⑤ details in Chapter 1.6.1; ⑥ details in Chapter 1.5.2.
The symbol "?" denotes possible changes during pregnancy considered in this thesis.

Stressors may arise in the external or internal environments. Stressors are classified into two types: social/emotional and physical stressors. Forced swimming and restraint belong to the former, and painful stress or footshock belong to the latter. Also from the external environment are micro-organisms, which if they invade the body and stimulate an immune response, activate the HPA axis through signalling by immune cells. Stressors within the body, for example hypoxia or hypotension, also trigger HPA activation.

1.2 Anatomy of HPA axis

The HPA axis consists of the hypothalamus, anterior pituitary and adrenal cortex.

1.2.1 The hypothalamus and the PVN

The hypothalamus is a region of the brain that controls an immense number of bodily functions. It is located in the middle of the base of the brain, and encapsulates the ventral portion of the third ventricle. There are 16 nuclei distinguished in the rat hypothalamus. The anterior hypothalamus includes the periventricular hypothalamic nucleus, suprachiasmatic nucleus, anterior hypothalamic nucleus, supraoptic nucleus (SON), PVN and retrochiasmatic area. The PVN lies at the caudal portion of the anterior hypothalamus, and contains CRH and vasopressin parvocellular neurones projecting to the median eminence and magnocellular vasopressin and oxytocin neurones projecting to the posterior pituitary or centrally (Michael & Freeman 2000). The SON contains magnocellular oxytocin neurones and vasopressin neurones in the dorsal and ventral portion of the nucleus respectively, all projecting to the posterior pituitary (Michael & Freeman 2000). The middle hypothalamus contains the arcuate nucleus, ventromedial nucleus, dorsomedial nucleus, and perifornical nucleus.

The median eminence is a tiny structure in the hypothalamus about 1.7-2.0 x 0.7-1.2 x 0.2-0.3 mm in rats, containing the efferent neurosecretory fibres of the hypothalamus that regulate the anterior pituitary or project to the posterior pituitary. The internal layer contains the fibres mainly from the magnocellular oxytocin and vasopressin

neurones in the PVN and SON, terminating in the posterior pituitary. The external layer contains the fibres originating from various hypothalamic nuclei including parvocellular CRH and vasopressin neurones and these neurone terminals abut on the pericapillary space surrounding the fenestrated capillaries of the primary pituitary portal plexus in the external zone of the median eminence and the hormones are released into the hypothalamic portal blood to get to the anterior pituitary (Holmes *et al.* 1986; Michael & Freeman 2000).

1.2.2 Anatomy of the pituitary gland

The pituitary gland, also known as the *hypophysis*, is a roundish organ that lies in the hypophyseal fossa of the sphenoid bone flanked on either side by the cavernous sinus and above by the optic chiasm, and is connected to the hypothalamus by the pituitary stalk. The human pituitary gland can be divided into two parts, the posterior pituitary is a direct extension of the central nervous system, whereas the anterior pituitary is derived from Rathke's pouch. But in rodents, the pituitary can be divided into three parts: the anterior (pars distalis), the intermediate (pars intermedia) and the posterior (pars nervosa) pituitary (Figure 1.2) (Michael & Freeman 2000).

1.2.2.1 The anterior pituitary

The anterior pituitary is formed of several cell types producing a range of hormones. An essential component in the organisation and function of the anterior pituitary is the close relationship between the cells and the capillary bed. Ready access to the circulation promotes uptake of the substrates for hormone synthesis and allows efficient release of hormones into the systemic circulation. The activity of the cells of the anterior pituitary is controlled by releasing factors present in the capillary blood. The releasing factors are produced by hypothalamic neurones and released into the hypothalamo-hypophyseal portal circulation, i.e the secretion of ACTH from the anterior pituitary is stimulated by CRH and vasopressin. There are five distinct types of endocrine cells, distributed either throughout the anterior pituitary or localised to particular parts. Corticotrophs are found throughout the anterior lobe in the rodents, secreting ACTH, β-lipotrophin, α-melanocyte stimulating hormone and β-endorphin

Figure 1.2 Anatomy of the pituitary

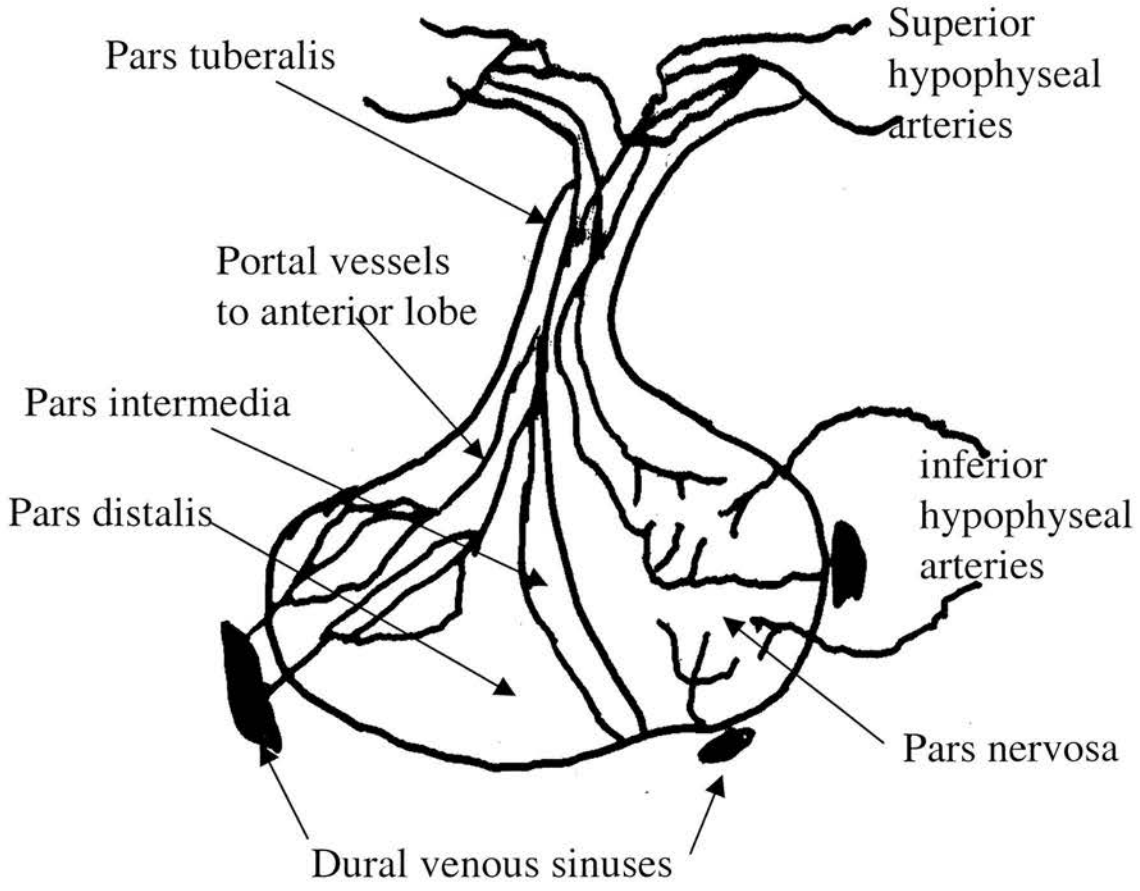


Figure 1.2 Anatomy of the pituitary

which are all products of the proopiomelanocortin (POMC) gene (Lowry *et al.* 1980; Chretien & Seidah 1981; Lundblad & Roberts 1988; King & Baertschi 1990). The other cells produce growth hormone, thyroid stimulating hormone, prolactin, follicle stimulating hormone and luteinising hormone (Michael & Freeman 2000).

1.2.2.2 The intermediate and posterior pituitary

The cells of the intermediate lobe also produce POMC-derived peptides (Michael & Freeman 2000). The posterior lobe or neurohypophysis contains terminals of

magnocellular SON and PVN neurones, releasing either oxytocin or vasopressin (Michael & Freeman 2000).

1.2.2.3 Blood supply

The pituitary gland derives its blood supply from the superior and inferior hypophyseal arteries that are connected by a portal system of small vessels and capillaries. There are specialised vascular structures located in the median eminence of the hypothalamus that consist of short terminal arterioles draining into portal veins that course down the pituitary stalk to join the sinusoidal capillaries of the anterior lobe. Releasing factors from the hypothalamus enter small openings in the capillaries to flow from the hypothalamus to the anterior pituitary (Michael & Freeman 2000).

1.2.3 Adrenal glands

The adrenal glands are paired triangular shaped structures located immediately rostral to the kidneys to which they are loosely attached. Each adrenal gland consists of two parts, the outer cortex and the inner medulla. Adrenal cortical tissue comprises large glandular cells arranged together in what appear to be loose cords separated by sinusoids. Three distinct zones are recognised: the inner layer zona reticularis and the middle layer zona fasciculata synthesise glucocorticoids and sex hormones, and the outermost layer zona glomerulosa synthesises mineralocorticoids. All adrenal cortical hormones are steroid hormones that are released as required. The major mineralocorticoid is aldosterone. The major glucocorticoids include cortisol in many species and corticosterone in rats. In contrast, the adrenal medulla comprises modified nerve cells known as chromaffin cells. They are equivalent to sympathetic postganglionic neurones. They synthesise and secrete two closely related hormones, adrenaline and noradrenaline. The adrenal medulla functions essentially as an endocrine adjunct to the sympathetic nervous system for rapid stress response. Release of adrenaline and noradrenaline are under the control of pre-ganglionic fibres which fire when the sympathetic nervous system is activated, for example during exercise, cold stress or haemorrhage (DeGroot 1995).

1.3 Stimulatory factors of ACTH secretion

1.3.1 CRH

1.3.1.1 CRH characterisation

The major stimulatory regulator of pituitary ACTH secretion is CRH (Figure 1.1) (Antoni 1986a). This 41 amino acid was characterised by Vale and his colleagues in 1981 from extracts of sheep hypothalamic fragments (Vale *et al.* 1981). Subsequently, rat CRH was isolated and the primary structure of rat CRH was established to be: H-Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-Ile-NH_2. The close structural relationship between rat and ovine hypothalamic CRH is indicated by an 83% sequence homology (Rivier *et al.* 1983). Subsequently, the cDNA of the CRH gene was cloned (Furutani *et al.* 1983; Shibahara *et al.* 1983). Rat CRH is identical to human CRH, and differs from ovine CRH by seven amino acids. Mammalian CRH has homologies with two peptides found in lower animal forms, the peptides sauvagine from frog and urotensin from fish. Both have potent CRH activity (Okawara *et al.* 1988; Stenzel-Poore *et al.* 1992). A mammalian CRH related peptide, urocortin, is related to urotensin (63% sequence identity) and CRH (45% sequence identity). Synthetic urocortin evokes secretion of ACTH both *in vitro* and *in vivo,* binds and activates type 1 CRH receptors (the subtype expressed by pituitary corticotrophs) and type 2 CRH receptors (Vaughan *et al.* 1995).

1.3.1.2 Distribution of CRH

The PVN of the hypothalamus was first convincingly implicated as the source of hypophysiotropic CRH activity in the lesion studies (Makara *et al.* 1981). The majority of CRH neurosecretory cells are located within the dorsal medial parvocellular subdivision of the PVN (Swanson & Kuypers 1980; Swanson *et al.* 1983). These neurones project their axons to the capillary plexus in the external zone of the median eminence, from where the CRH is release into the hypothalamo-hypophysial portal circulation (Antoni 1986a). About 2,000 CRH immunoreactive cells are distributed throughout the PVN, a majority (80%) of the cells are localised in the parvocellular division and a smaller number (about 15%) are found in the

magnocellular division. Most of the CRH stained fibres in the external zone of the median eminence appear to arise in the PVN (Swanson *et al.* 1983).

CRH has a broad distribution throughout the brain and periphery and has been shown to be present in certain human tumors (Parkes *et al.* 1993b). A series of cell groups in the basal telencephalon, hypothalamus, and brain stem that are known to play a role in the mediation of autonomic responses contain CRH immunoreactive neurones: the central nucleus of the amygdala, substantia innominata, bed nucleus of the stria terminalis, medial and lateral preoptic areas, lateral hypothalamic area, central gray, laterodorsal tegmental nucleus, locus coeruleus, parabrachial nucleus, dorsal vagal complex, and regions containing the A1 and A5 catecholamine cell groups. Scattered CRH-stained cells are also found throughout most areas of the cerebral cortex, dorsal parts of the dentate gyrus and Ammon's horn (Swanson *et al.* 1983).

1.3.1.3 Physiological functions of CRH

Brain CRH is involved in coordinating the pituitary and autonomic nervous system responses to stress (Brown *et al.* 1985). CRH synthesised in the parvocellular PVN of the hypothalamus is transported in the axons of these neurones, projecting to the median eminence and released into the portal vessels (King & Baertschi 1990). The concentration of CRH in the portal blood of anaesthetised rats is about 0.1 nM, which is identical to that necessary for significant stimulation of ACTH secretion by CRH *in vitro* (Vale *et al.* 1981; Gibbs & Vale 1982). Substantial studies have shown that CRH originating from neurones within the PVN is the predominant regulator of stress-induced ACTH secretion. These include PVN lesion (Baertschi *et al.* 1983; Bruhn *et al.* 1984a), synthetic ovine CRH administration *in vitro* (Gibbs *et al.* 1983), and intravenous rabbit antiserum to ovine CRH injection (Rivier *et al.* 1982) studies. Intracerebroventricular injections of ovine CRH results in a 50-100% increase in POMC mRNA expression in the anterior pituitary (Bruhn *et al.* 1984b). Intravenous injections of ovine CRH also increase POMC mRNA expression in the anterior pituitary (Bruhn *et al.* 1984b) and plasma ACTH levels in rats (Hylka *et al.* 1984). Intravenous administration of rabbit antiserum to ovine CRH markedly reduces the CRH-induced rise of plasma ACTH in intact nonstressed rats, and blocks the ether stress- or adrenalectomy- induced increases in ACTH levels by more than 75% (Rivier *et al.* 1982). A bilateral ablation of the PVN results in a parallel 75% reduction

of both stalk-median eminence CRH-like immunoreactivity and plasma ACTH levels (Bruhn *et al.* 1984b). The presence of CRH receptor in the corticotrophs (Potter *et al.* 1994) and attenuated lipopolysaccharide-induced ACTH secretion by intravenous injection of CRH receptor antagonist (Watanabe *et al.* 1994), α-helical CRH, also indicate a role of CRH in stimulating ACTH secretion.

CRH is also involved, within the brain, in physiological regulation other than of ACTH secretion, such as decreasing food intake and body-weight gain (Hotta *et al.* 1991).

1.3.1.4 Regulation of CRH synthesis and release

The mechanisms controlling the expression and release of CRH by PVN neurones are complex, with a number of stimulatory factors (Antoni 1986a) including catecholamines (Guillaume *et al.* 1987; Plotsky *et al.* 1989), cytokines (Barbanel *et al.* 1990; Rivier & Rivest 1993), serotonin (Bagdy *et al.* 1989), histamine (Calogero 1995), acetylcholine (Jones *et al.* 1987), angiotensin II (Suda *et al.* 1985), opioids (Buckingham & Cooper 1986) and inhibitory factors, including glucocorticoids and gamma-aminobutyric acid (GABA) (Jones *et al.* 1987), being implicated. CRH neurones also receive dense adrenergic and noradreneragic innervation from the brain stem, and adrenergic receptors including α1-, α2-, β1-, β2 have been identified in the PVN (Leibowitz *et al.* 1982; Cummings & Seybold 1988; Al Damluji 1993).

Glucocorticoid receptors are localised in the parvocellular neurones of the PVN where CRH is produced (Liposits *et al.* 1987), which indicates the PVN is a direct site of glucocorticoid action. Basal CRH mRNA expression in the PVN is under the inhibitory influence of glucocorticoids, as shown by the marked increases in ir-CRH and CRH mRNA following adrenalectomy (Antoni 1986a; Young, III *et al.* 1986; Sawchenko 1987; Swanson & Simmons 1989). Corticosteroid feedback reduces CRH mRNA levels in a dose-dependent manner, although even prolonged administration of very high doses does not abolish CRH transcripts completely. Adrenalectomy increases hypothalamic CRH mRNA expression, while dexamethasone administration reverses this adrenalectomy effect (Jingami *et al.* 1985; Beyer *et al.* 1988; Imaki *et al.* 1991; Imaki *et al.* 1995) in the PVN to basal levels. Glucocorticoid feedback also

inhibits CRH gene expression in the PVN after stress, as adrenalectomy augmented and dexamethasone pretreatment inhibited both CRH hnRNA and mRNA induction in the PVN after the acute stress (Imaki *et al.* 1995).

The activation of the PVN CRH neurones during acute stress is usually followed by variable increases in CRH synthesis as indicated by increases in CRH mRNA expression as well as CRH release (Suda *et al.* 1988b; Harbuz & Lightman 1989; Imaki *et al.* 1991). CRH mRNA levels in the PVN were determined after exposure to a variety of acute physical and psychological stresses. CRH mRNA in the PVN measured by *in situ* hybridisation increases in response to intraperitoneal injection of hypertonic saline, restraint and swim stress but not to cold stress which suggests that different afferent pathways and hypothalamic neurotransmitters may be involved in mediating the hypothalamic response to different physical and psychological stresses (Harbuz & Lightman 1989; Imaki *et al.* 1995). Many types of chronic stress also enhance CRH mRNA expression (Imaki *et al.* 1991; Bartanusz *et al.* 1993; Kiss & Aguilera 1993; Lightman & Harbuz 1993).

CRH secretion is also regulated by itself. By intraventricular injection, CRH shows an augmentation effect of stress on CRH release (Ono *et al.* 1985a) and increases CRH mRNA expression in the PVN (Parkes *et al.* 1993a).

Some evidence indicates that CRH gene expression in the PVN is also regulated by sex steroids. Estrogen stimulates CRH mRNA expression in the PVN, whereas progesterone reverses the effect of estrogen *in vivo*. Measured by *in situ* hybridisation, after chronic sex steroid replacement, CRH mRNA in the PVN is increased by 2-fold in the 17β-estradiol-replaced ovariectomised rhesus monkey compared with untreated ovariectomised controls, but is not different compared with the controls after 17β-estradiol plus progesterone replacement (Roy *et al.* 1999). *In vitro*, estradiol, oestriol and oestrone potentiate the effect of acetylcholine or 5-hydroxytryptamine on the production of CRH from the isolated hypothalamus, but progesterone, testosterone, androsterone and androstenedione have no such effects (Buckingham 1982).

1.3.1.5 CRH receptors

1.3.1.5.1 Characterisation of CRH receptors

CRH as a regulator of ACTH secretion by the pituitary corticotrophs acts through specific plasma membrane receptors (Figure 1.1). Two major subtypes of CRH receptor, differing in their pharmacological properties and distribution, and the products of distinct genes, have been identified. CRH receptor type 1 (CRHR1), the main subtype in pituitary and brain, and CRH receptor subtype 2 (CRHR2).

The molecular weights of rat and mouse brain CRHR1s are 76-80 kDa and 79-83 kDa respectively, whereas the molecular weights for CRHR1 from rat and mouse pituitary are 59-72kDa (Radulovic *et al.* 1998). Other studies also show that brain and pituitary CRH receptors have similar pharmacological characteristics but different molecular weights (brain, 58 kDa; anterior pituitary, 75 kDa) (Grigoriadis & De Souza 1989a; Grigoriadis & De Souza 1989b).

CRHR1 cDNA was cloned from a human corticotropic tumor library (Chen *et al.* 1993) and from rat pituitary (Perrin *et al.* 1993). This encodes a 415-amino acid protein comprising seven putative membrane-spanning domains and is structurally related to the calcitonin/vasoactive intestinal peptide/growth hormone-releasing hormone subfamily of G protein-coupled receptors. The receptor binds rat/human CRH with high affinity (Kd = 3.3 ± 0.45 nM) and is functionally coupled to adenylate cyclase (Chen *et al.* 1993). The rat CRHR1 is 97% identical at the amino acid level to the human pituitary tumor CRH receptor, differing by only 12 amino acids.

CRHR2 cDNA was cloned from rat and human brain (Lovenberg *et al.* 1995; Liaw *et al.* 1996), and encodes a 411-amino acid protein with approximately 70% identity to CRHR1 over the entire coding region. For the CRHR2 subtype, at least 2 splice forms with different 5'-coding sequences (CRHR2α and CRHR2β) have been identified (Lovenberg *et al.* 1995; Palchaudhuri *et al.* 1999).

The activation of these receptors by CRH and known CRH-like agonists stimulates cAMP production. In response to CRH and the CRH-related peptides sauvagine and urotensin, adenylate cyclase activity is stimulated in a dose-dependent manner. The

rank order of potencies of agonist peptides are CRH = sauvagine = urocortin = urotensin at CRHR1, and urocortin = sauvagine > urotensin > CRH at CRHR2 (Smart *et al.* 1999).

1.3.1.5.2 Distribution of CRH receptors

In the pituitary, using radioiodinated ovine CRH, the distribution of CRH binding sites was found mainly in the clusters of corticotrophs in the anterior lobe and the opiomelanocortin-producing cells in the intermediate pituitary (De Souza *et al.* 1984). Moderate levels of CRHR1 mRNA expression are detected throughout the intermediate lobe and in corticotrophs in the anterior lobe (Potter *et al.* 1994) (Chalmers *et al.* 1995).

CRHR1 is also expressed in discrete brain areas including the cerebral cortex, several regions related to the limbic system and peripheral tissues (De Souza *et al.* 1985; Udelsman *et al.* 1986; Millan *et al.* 1987; Primus *et al.* 1997; Baigent & Lowry 2000a). By *in situ* hybridisation CRHR1 expression in the forebrain is dominated by widespread signal throughout all areas of the neo-, olfactory, and hippocampal cortices, and subcortical limbic structures in the septal region and amygdala. Low levels of expression are seen in a few discrete ventral thalamic and medial hypothalamic nuclei. CRHR1 expression in hypothalamic neurosecretory structures, including the PVN and median eminence, is generally low. In the brainstem, certain relay nuclei associated with the somatic (including trigeminal), auditory, vestibular, and visceral sensory systems, constitute prominent sites of CRHR1 mRNA expression. In addition, high levels of this transcript are present in the cerebellar cortex and deep nuclei, along with many precerebellar nuclei (Potter *et al.* 1994).

CRHR1 mRNA is also found in thymus, spleen, thecal cells of mature follicles and moderately in small antral follicles (Asakura *et al.* 1997; Baigent & Lowry 2000a).

In the brain CRHR2 mRNA is widely distributed including the olfactory bulb, amygdaloid nuclei, the PVN and SON of the hypothalamus, and the bed nucleus of the stria terminalis. But there is no CRHR2 expression detected in the pituitary (Chalmers *et al.* 1995; Primus *et al.* 1997).

This heterogeneous distribution of CRHR1 and CRHR2 mRNAs suggests distinctive functional roles for each receptor in CRH-related systems. The CRH1 receptor may be regarded as the primary neuroendocrine pituitary CRH receptor and important in cortical, cerebellar and sensory roles of CRH. The anatomical distribution of CRH2 receptor mRNA indicates a role for this receptor in hypothalamic neuroendocrine, autonomic and general behavioural actions of central CRH.

1.3.1.5.3 The cAMP pathway

The action of CRH on ACTH secretion is mediated by CRHR1: after binding to its receptors on the corticotrophs, CRH stimulates ACTH secretion through the cAMP pathway.

cAMP is a classical second messenger, initiating a cascade of intracellular events leading to a physiological response, synthesised from ATP on the inner surface of the plasma membrane. Fleischer and colleagues first found that cAMP is involved in the release of ACTH from the anterior pituitary (Fleischer *et al.* 1969). CRH binding to the CRH receptors on the corticotroph stimulates the guanine nucleotide-binding protein (Gs), and subsequently, adenylate cyclase (Perrin *et al.* 1986; Battaglia *et al.* 1987) is activated by Gs to convert Mg^{2+}ATP to cAMP. cAMP then activates protein kinase A (PKA) and is degraded by cAMP-dependent phosphodiesterase. PKA modulates POMC gene transcription, translation, processing and/or ACTH release (Aguilera *et al.* 1986; King & Baertschi 1990), although the substrates of PKA are not fully elucidated. CRH increases secretion of ACTH from corticotrophs by stimulating exocytosis with an increase in cell surface area accompanied by a decrease in patches of ACTH immunostaining within the cells (Westlund *et al.* 1985; Kubba & McNicol 1987).

The release of ACTH induced by CRH from the corticotrophs is largely dependent on cAMP-mediated stimulation of Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels (Luini *et al.* 1985; Aguilera *et al.* 1986; Guerineau *et al.* 1991). CRH depolarises the cell, increasing firing frequency in spontaneously active cells and inducing action potentials in quiescent cells. The CRH-induced membrane depolarisation is due to in part inhibition of large-conductance Ca^{2+}-activated K^{+}-channels (BK-channel) by PKA.

Among the PKA targets in AtT20 cells, L-type Ca^{2+} channels and the BK-channels are two significant functional targets. PKA activates L-type calcium channels increasing the probability of channel opening and prolonging the opening state of L-type channels upon depolarisation (Wang *et al.* 1993) through PKA-dependent phosphorylation of either the channel subunits directly or other closely associated channel proteins (Rougon *et al.* 1989; Charnet *et al.* 1995; Puri *et al.* 1997). In AtT20 cells PKA-mediated phosphorylation inhibits the opening of BK-channels (Shipston *et al.* 1996). The resulting Ca^{2+} influx raises the cytosol Ca^{2+} and triggers the calcium sensor element of the exocytotic apparatus, stimulating the secretion of ACTH. Although there is evidence that cAMP-mediated phosphorylation acts in synergy with Ca^{2+} to stimulate ACTH release, the exact mechanism(s) are not known (Guild 1991).

1.3.1.5.4 Regulation of anterior pituitary CRHR1 gene expression

CRHR1 gene expression is evidently regulated by CRH and vasopressin. Intravenous and subcutaneous infusion of CRH for 48 h in intact rats caused the pituitary CRH receptor concentration to decrease (Wynn *et al.* 1988). Minipump infusion of CRH caused a decrease in the anterior pituitary CRH receptor concentration. Exogenous vasopressin infusion reduces anterior pituitary CRHR1 contents in Brattleboro (di/di) rats. In S-D strain rats, CRH or vasopressin infusion also reduces CRH contents in the anterior pituitary and vasopressin potentiates CRH effect when infused together (Holmes *et al.* 1987). When the parvocellular vasopressinergic system is activated by repeated restraint stress, there is a significant enhancement in the ability of CRH to decrease anterior pituitary CRH receptors, however, activation of the magnocellular vasopressinergic system by water deprivation has no effect on the anterior pituitary CRH receptor concentration (Hauger & Aguilera 1993).

In cultured rat anterior pituitary cells, CRH and vasopressin treatment decreases CRHR1 mRNA levels in a time- and concentration-dependent manner (Sakai *et al.* 1996; Pozzoli *et al.* 1996). *In vitro* incubation of quartered pituitaries with vasopressin or CRH or both peptides (Holmes *et al.* 1987) confirms that vasopressin synergises with the effect of CRH on CRHR1 downregulation.

In the presence of both vasopressin and CRH, vasopressin potentiates the CRH effect on CRHR1 mRNA expression (Pozzoli *et al.* 1996). However, *in vivo* a single vasopressin injection, as well as repeated injection for 14 day, increase CRHR1 mRNA expression in the anterior pituitary (Rabadan-Diehl *et al.* 1996).

During prolonged or acute immobilisation, anterior pituitary CRH receptor content is down- regulated (Hauger *et al.* 1988; Hauger *et al.* 1990; Makino *et al.* 1995a). However, changes in expression can be quite rapid. *In situ* hybridisation studies showed biphasic changes in CRHR1 mRNA expression in the anterior pituitary after immobilisation stress with a decrease by 2 h, increase at 4 and 8 h after the initiation of the stress, and a return to near basal levels by 12 and 18 h. A different pattern was observed, with a decrease by 4 h and levels similar to controls by 12 and 18 h after a single intraperitoneal injection of hypertonic saline (1.5 M NaCl). Binding autoradiography shows significant increases in pituitary CRH binding 4, 10, and 12 h after immobilisation stress, but significant decreases 4, 12, and 18 h after intraperitoneal hypertonic saline injection. In contrast, repeated immobilisation or intraperitoneal hypertonic saline for 8 or 14 days increases pituitary CRHR1 mRNA expression (Rabadan-Diehl C *et al.* 1996).

In both adrenalectomised rats with corticosterone pellet replacement (39 mg; adrenalectomy with corticosterone replacement rats) and control, acute (2 h) and repeated (2 h daily for 14 days) immobilisation stress (which produce a large increase in plasma corticosterone in control rats) decreases CRHR1 mRNA in the anterior pituitary. However, adrenalectomised rats given corticosterone consistently have higher levels of CRHR1 mRNA in the anterior pituitary than control rats after stress (Makino *et al.* 1995a).

Glucocorticoids or dexamethasone decrease in a dose-dependent manner CRH binding in the anterior pituitary *in vivo* and *in vitro* (Childs *et al.* 1986). In cultured rat anterior pituitary cells, dexamethasone and corticosterone cause a dose- and time-dependent decrease in CRHR1 mRNA levels (Pozzoli *et al.* 1996; Sakai *et al.* 1996). Elevations in plasma corticosterone (in the range altered during acute stress) by subcutaneous injection of corticosterone (10 mg), caused CRHR1 mRNA expression to return to near basal values by 6 h, after a 52% and 39% decrease at 2 h and 4 h

(Ochedalski *et al.* 1998). Chronic corticosterone injection (10 mg/rat.day, for 7 days) also decreases CRHR1 mRNA in the anterior pituitary.

Adrenalectomy also decreases CRHR1 mRNA expression in the anterior pituitary (Makino *et al.* 1995a), but pituitary CRHR1 mRNA levels are not affected by long term adrenalectomy (Luo *et al.* 1995; Rabadan-Diehl *et al.* 1997c). The CRHR1 mRNA level in anterior pituitary falls to 20% of the control level 1 day after adrenalectomy and returned to the control level after 14 days (Sakai *et al.* 1996). After adrenalectomy, the concentration of CRH receptor in the pituitary decreases (Wynn *et al.* 1985). This decrease is not entirely due to occupancy by endogenous CRH, since high dose infusions of CRH (300-500 ng/min) for 30 min before pituitary membrane preparation reduced CRH-binding sites by only 40%, while CRH receptor concentration in the pituitary decreases by over seventy percent 2 days after adrenalectomy, which increases CRH secretion. Such decreases in CRH receptors in adrenalectomised rats are prevented by dexamethasone treatment (Wynn *et al.* 1985) and completely prevented by medial basal hypothalamic lesioning, indicating that receptor down-regulation is dependent on the release of CRH or/and other hypothalamic factors(Wynn *et al.* 1988).

A single injection of CRH decreases CRHR1 mRNA levels in adrenalectomised rats, but not in glucocorticoid-replaced adrenalectomised rats (Rabadan-Diehl C *et al.* 1996). In the absence of glucocorticoids in adrenalectomised rats, acute stress results in prolonged CRHR1 mRNA loss, suggesting that interactions between glucocorticoids and hypothalamic factors are critical for regulation of CRHR1 mRNA expression (Rabadan-Diehl *et al.* 1996). After a single or repeated injection of CRH, CRHR1 mRNA in the anterior pituitary decreased by 2 h, returning to basal values by 4 h, however, CRH injection in adrenalectomised rats decreased CRHR1 mRNA for up to 6 h, suggesting that glucocorticoids are permissive for the recovery of CRHR1 mRNA expression (Ochedalski *et al.* 1998).

In summary, CRH decreases CRHR1 mRNA expression in the anterior pituitary, and vasopressin potentiates this CRH effect. Glucocorticoids also decrease CRHR1 mRNA expression, but are permissive for the recovery of CRH-decreased CRHR1 mRNA expression.

1.3.2 Vasopressin

1.3.2.1 Vasopressin characterisation and physiological roles

Vasopressin is produced in parvocellular CRH neurones of the PVN and magnocellular neurones of the PVN and SON as part of a larger precursor protein that is cleaved in the secretory granules to produce vasopressin, neurophysin and co-peptin (William 1987) (Figures 1.1 and 1.3). Vasopressin synthesised in parvocellular neurones of the PVN is responsible for stimulating ACTH secretion, after transport via nerve fibres in the external zone of the median eminence and release into the capillaries of the portal system, whereas vasopressin produced in magnocellular neurones of the PVN and SON is transported to the pituitary neural lobe and secreted into the peripheral circulation, in response to osmotic and other stimuli (Antoni 1986a; Holmes *et al.* 1986; Antoni *et al.* 1990). Osmotic activation of the magnocellular vasopressin system is accompanied by reduced responsiveness of the corticotrophs. This effect is not due to increased glucocorticoid feedback since similar blunted ACTH responses to acute immobilisation stress are observed in adrenalectomised rats receiving corticosterone replacement. Similarly, hypothalamic CRH is not deficient as the ir-CRH content in the median eminence and its release under stress are similar in control and water-deprived rats. These studies suggest that the magnocellular vasopressinergic system does not play an important role in the regulation of ACTH secretion during chronic osmotic stimulation (Aguilera *et al.* 1993). However, some evidence also demonstrates that vasopressin from magnocellular neurones, released by axons in the median eminence, participates in ACTH secretion regulation (Antoni 1993).

Figure 1.3 The hypothalamic sources of vasopressin input to the pituitary

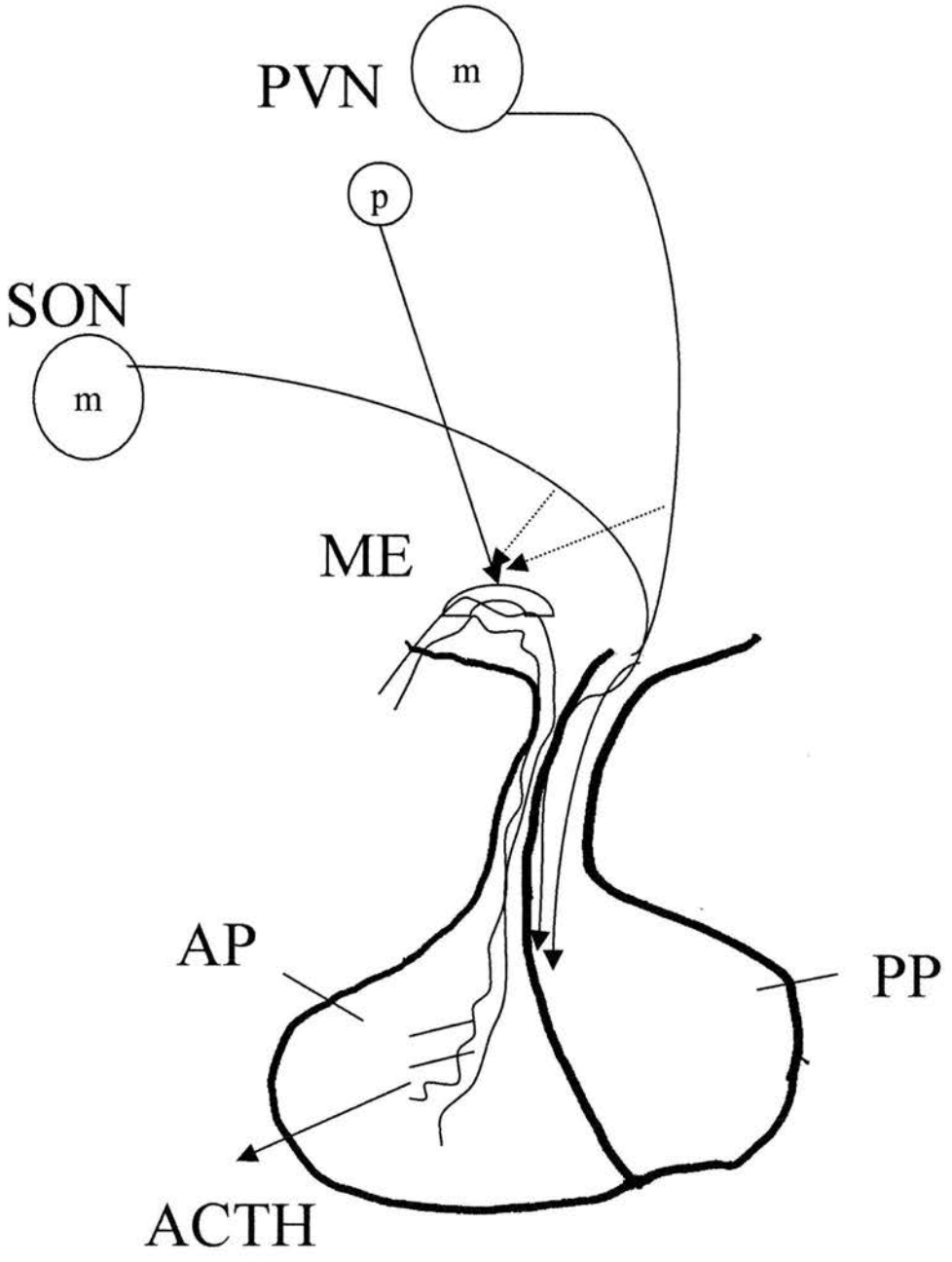


Figure 1.3 The hypothalamic sources of vasopressin input to the pituitary.
Vasopressin of parvocellular origin is released into the portal blood to regulate ACTH secretion from the anterior pituitary. Vasopressin of magnocellular origin is transported to the posterior lobe, stored there and released in response to osmotic stimulation. AP= anterior pituitary; ME= median eminence; PP= posterior pituitary; m= magnocellular neurones; p= parvocellular neurones. small amount of vasopressin may be leaked to the median eminence when transported from magnocellular neurones to the posterior pituitary.

Vasopressin and CRH are co-localised in parvocellular neurones of the PVN of the hypothalamus and are capable of potentiating each others' action on ACTH secretion by freshly prepared anterior pituitary fragments or cells *in vitro* (Makara 1992). About 50% of the parvocellular CRH perikarya contain vasopressin under basal conditions (Whitnall *et al.* 1985) and the proportion of vasopressin immunoreactive- and mRNA-positive neurones increases following adrenalectomy and chronic stress paradigms associated with hypersensitivity of the HPA axis (Whitnall 1988; Whitnall 1989; de Goeij *et al.* 1992c; Bartanusz *et al.* 1993; Paulmyer-Lacroix *et al.* 1994).

Vasopressin alone is a less effective stimulator of ACTH secretion in rodents, but it is reported to produce greater ACTH biosynthetic and secretory responses than does CRH in sheep anterior pituitary cells (van de Pavert *et al.* 1997). The actions of vasopressin on ACTH secretion from corticotrophs are mediated by plasma membrane receptors belonging to the guanyl nucleotide binding protein (G-protein family), vasopressin receptor subtype 1b (Jard *et al.* 1986) (see Chapter 1.3.2.3). CRH is a potent inducer of POMC mRNA transcription, while vasopressin alone does not increase expression of this gene (Antoni 1993) or it even inhibits basal and CRH-stimulated POMC transcription *in vitro* (Levin *et al.* 1989). However, one recent study shows that vasopressin may have a stimulatory effect on POMC gene expression by activating POMC promoter activity via PKC (Aoki *et al.* 1997). A vasopressin antagonist decreases the plasma ACTH levels in ether-stressed rats (Rivier & Vale 1983). Vasopressin antiserum microinjection into the third ventricle decreases the elevation of plasma ACTH induced by ether and restraint stresses (Linton *et al.* 1985). This inhibitory action of the vasopressin antiserum on ACTH release may be mediated intrahypothalamically by blocking the stimulatory action of vasopressin on CRH neurones and/or also in part by direct blockade of the stimulatory (augmentation) action of vasopressin on the pituitary (Ono *et al.* 1985b).

1.3.2.2 Regulation of vasopressin secretion and gene expression in the hypothalamus

Parvocellular vasopressin secretion is regulated by different stress paradigms. Release of vasopressin within the PVN is significantly increased in response to emotional stressors (Wotjak *et al.* 1996). During emotional stress vasopressin gene transcription in the PVN and SON increases (Priou *et al.* 1993). Intraperitoneal injection of hypertonic saline for 14 days causes enhanced parvocellular activity as indicated by increases in vasopressin immunostaining in the external zone of the median eminence (Kiss & Aguilera 1993): this may be due to the painful stress of the injection. Chronic immobilisation stress exposure results in a significant increase in the average cellular level of CRH and vasopressin mRNAs in the parvocellular PVN, and the number of vasopressin-expressing parvocellular neurones is approximately doubled (Bartanusz *et al.* 1993; Herman *et al.* 1995). Acute ether stress provokes rapid and transient increases in CRH hnRNA: this peaks at 5 min and is followed by a delayed upregulation of vasopressin hnRNA (120 min) in parvocellular neurones of the PVN (Kovacs & Sawchenko 1996). Parvocellular vasopressin hnRNA responses to a single hypertonic saline injection are slower and more prolonged than for CRH, and vasopressin mRNA levels increase by 4 h and remain elevated 12 h later. Parvocellular vasopressin hnRNA expression is at basal levels after 14 days hypertonic saline injection, but vasopressin mRNA levels remain elevated as during acute stimulation (Ma & Aguilera 1999b).

It has been revealed that glucocorticoid negative feedback during stress selectively targets vasopressin transcription in the parvocellular neurones that is likely mediated by interaction of glucocorticoid receptors and immediate-early gene products (Kovacs 1998). Acute immobilisation causes a significant increase in CRH, but not vasopressin, mRNA levels in the parvocellular PVN in sham rats, while this acute immobilisation increases both CRH and vasopressin mRNA levels in the PVN in the adrenalectomised rats with a moderate dose of corticosterone replacement. These data indicate that PVN vasopressin mRNA levels are more sensitive to glucocorticoid negative feedback than are the levels of CRH mRNA (Makino *et al.* 1995b). After adrenalectomy, levels of vasopressin hnRNA show biphasic changes: grain density increases 11.5- fold over sham-operated controls by 6 h, declines to 2-fold by 18 h, to increase again to 10- and 20-fold by 48 and 72 h, respectively. Vasopressin hnRNA

levels increased only transiently by 15 min after corticosterone administration to adrenalectomised rats, and then decreased below basal (near sham-adrenalectomy levels) by 2 h. The data show that in normal conditions the responsiveness of parvocellular neurones to stress is under marked inhibition by the low resting levels of glucocorticoids (Ma & Aguilera 1999a).

In contrast, vasopressin expression in the magnocellular neurones is regulated by osmotic changes. Vasopressin mRNA levels measured by *in situ* hybridisation within magnocellular neurones of the SON and PVN increase after chronic water deprivation (Meeker *et al.* 1991; Kiss *et al.* 1994). Intraperitoneal injection of hypertonic saline for 14 days causes increases in plasma vasopressin, and marked increases in vasopressin mRNA and ir-vasopressin in magnocellular cell bodies in the hypothalamus (Kiss & Aguilera 1993). Increases in vasopressin mRNA and ir-vasopressin in magnocellular cell bodies depend on the intensity and duration of the stimulation with a range of stimuli (Burbach *et al.* 1984; Meeker *et al.* 1991).

In summary, vasopressin expression in the parvocellular neurones in the PVN is increased by physical or emotional stressors and is inhibited by glucocorticoid negative feedback. In contrast vasopressin expression in the magnocellular neurones in the PVN and SON is regulated by osmotic challenges.

1.3.2.3 Vasopressin receptors

The actions of vasopressin are mediated through plasma membrane receptors (Figure 1.1) belonging to the guanyl nucleotide binding protein (G protein) superfamily consisting of seven hydrophobic transmembrane α-helices joined by alternating intracellular and extracellular loops, an extracellular N-terminal domain, and a cytoplasmic C-terminal domain. So far three receptor subtypes encoded by different genes have been identified and cloned: V1a receptors, V1b receptors and kidney V2 receptors.

1.3.2.3.1 Characterisation of vasopressin receptors

The V1b receptor was first identified on the basis of its pharmacology (Jard *et al.* 1986), and characterised in the anterior pituitary (Lolait *et al.* 1995), where it links to

phospholipase C to regulate vasopressin-mediated ACTH release by potentiating the effect of CRH.

The cDNA encoding the V1b receptor has been isolated and characterised from human, rat, and mouse (de Keyzer *et al.* 1994; Lolait *et al.* 1995; Ventura *et al.* 1999). The structure of the V1b receptor gene contains at least three exons and two introns (Rabadan-Diehl *et al.* 2000). The human V1b receptor comprises a 424 amino acids sequence and seven putative transmembrane domains and an overall sequence homology of 45, 39, and 45% with sequences of V1a, V2 and oxytocin receptors, respectively (Sugimoto *et al.* 1994). The rat V1b receptor is a protein of 421- or 425-amino acid that has 37-50% identity with the V1a and V2 receptors, having the highest identity with the human V1b (81%) (Saito *et al.* 1995).

The V1b receptor gene has two splice variants in rats, as suggested by Northern blot analysis. A 363 bp ^{32}P-labelled fragment of the rat V1b receptor cDNA coding sequence revealed two mRNA bands of about 3.7 and 3.2 Kb, whereas a probe directed to the 5' untranslated region (5'-UTR) recognised only the 3.7 Kb band. The 3.7 Kb splice variant encodes a bioactive vasopressin receptor, but the function of the 3.2 Kb splice variant is not clear. However both splice variants are expressed in parallel during the manipulation of HPA axis activity (Rabadan-Diehl *et al.* 1995).

The V1a receptor purified from rat and pig liver has a molecular weight of 58-60 kD, mediating the vasoconstrictor and hepatic glycogenolytic action of vasopressin (Fishman *et al.* 1987; Estrada *et al.* 1991). The human V1a receptor has been cloned (Morel *et al.* 1992), and encodes a 418-amino acid polypeptide with seven putative transmembrane domains typical of G protein-coupled receptors. Its amino acid sequence identity with the rat liver V1a vasopressin receptor, the human and rat V2 vasopressin receptors, and the human oxytocin receptor is 72, 36, 37, and 45%, respectively (Thibonnier *et al.* 1994). Vasopressin action through the V1a receptor is mediated by activating phospholipase C, which increases phosphatidylinositol turnover to increase intracellular calcium. V1a receptor mRNA is localised in the liver, kidney, vascular smooth muscle, platelets, renal medulla, renal cortex, and in many brain areas including the hippocampal formation, central amygdala, dorsolateral septum, lateral hypothalamus, suprachiasmatic, ventromedial, dorsomedial, and

arcuate nuclei of the hypothalamus, nucleus of the solitary tract, cerebellum, spinal nucleus of the trigeminal tract, reticular formation, inferior olivary nucleus, and choroid plexus. Rare labelled cells are seen along the periphery of the posterior pituitary (Ostrowski *et al.* 1992).

The V2 receptor is predominantly expressed in the kidney and mediates the antidiuretic effect of vasopressin by regulating water re-absorption in the collecting duct and medullary thick ascending limb of the loop of Henle via the activation of adenylate cyclase. V2 receptor transcripts have not been detected in the liver or brain, but are present in high amounts in the inner and outer renal medulla, primarily associated with the collecting ducts in kidney (Ostrowski *et al.* 1992).

1.3.2.3.2 Distribution of the V1b receptor

The V1b receptor is expressed in the majority of pituitary corticotrophs (de Keyzer *et al.* 1994; Ventura *et al.* 1999), but not in the mouse corticotroph tumor cell AtT20 line (Ventura *et al.* 1999). Double V1b receptor and POMC mRNA *in situ* hybridisation detects that the majority of the V1b receptor expressing cells are POMC mRNA positive and only a few of them do not express the POMC gene (Lolait *et al.* 1995). These non-corticotroph V1b receptor- expressing cells are thought to be thyrotrophs because vasopressin is reported to act as a thyrotropin-releasing hormone (Michael *et al.* 1986). V1b receptors are not detected in the intermediate pituitary.

The V1b receptor is also located in neurones in multiple brain regions (Hernando *et al.* 2001): the olfactory bulb, forebrain (the piriform and tenia taecta), in hypothalamus (suprachiasmatic nucleus, arcuate nucleus), hippocampus, in midbrain (substantia nigra, red nucleus, entorhinal cortex and paphe nucleus), thalamus, dorsal motor nucleus of the vagus, the choroid plexus, lateral septum, bed nucleus of stria terminalis, accumbens nucleus, central nucleus of amygdala, nucleus of the solitary tract, area postrema, superior colliculus, and inferior olivary nuclei (Phillips *et al.* 1988; Hurbin *et al.* 1998; Vaccari *et al.* 1998). Recently, the V1b receptor mRNA was also detected in the magnocellular neurones in the PVN and SON of the hypothalamus (Hurbin *et al.* 1998).

A number of peripheral tissues, including kidney, thymus, heart, lung, spleen, uterus, pancreas, colon and breast, express V1b receptors (de Keyzer *et al.* 1994; Lolait *et al.* 1995).

1.3.2.3.3 The PI pathway

Vasopressin regulates ACTH secretion through stimulation of phosphatidyl inositol turnover, as suggested by measurements of the incorporation of ^{32}Pi or ^{3}H-inositol into membrane phospholipids in cultured anterior pituitary cells (Raymond *et al.* 1985; Guillon *et al.* 1987; Todd & Lightman 1987).

When vasopressin binds to V1b receptors on corticotrophs coupled through G protein to phospholipase C, phosphatidylinositol 4,5-biphosphate, a minor lipid constituent of the plasma membrane, is cleaved into inositol-1,4,5 trisphosphate (IP_3) and diacylglycerol. IP_3 diffuses to the cytoplasm to mobilise internal sequestered calcium stores leading to increased cytosolic free calcium then to the activation of calcium-dependent processes, and is degraded by a phosphatase to IP_2. Diacylglycerol remains within the plasma membrane where it activates protein kinase C (PKC) translocated from the cytoplasm to the plasma membrane by increased cytosolic free calcium. PKC phosphorylates some unknown proteins leading to increasing secretion and (or) ACTH biosynthesis. Diacylglycerol can also be hydrolysed to arachidonic acid by phospholipase A_2 and arachidonic acid is further metabolised into prostaglandins and thromboxanes through the cyclooxygenase pathway forming an inhibitor, or leukotrienes through the lipoxygenase pathway forming a stimulator of ACTH secretion (King & Baertschi 1990).

Calcium signalling is also involved in vasopressin stimulated ACTH secretion through the IP pathway. This involves the generation of IP_3 with consequent release of sequestered intracellular Ca^{2+} (Guillemette *et al.* 1987) and generation of diacylglycerol and activation of PKC with consequent extracellular Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels (Nishizuka 1984; Won *et al.* 1990). This increased intracellular Ca^{2+} then triggers ACTH secretion from the corticotrophs.

1.3.2.3.4 Potentiating effect of vasopressin on ACTH secretion

Although vasopressin is a weak secretagogue alone, it is able to potentiate the action of CRH on ACTH secretion from corticotrophs. In 1982 Gillies et al first reported that CRH effect on ACTH secretion is potentiated by several times by vasopressin in isolated primary anterior pituitary (Gillies *et al.* 1982). Simultaneous addition of arginine-vasopressin (10^{-10} M) to the incubation medium containing CRH (5.0×10^{-9} - 2.0×10^{-7} M) enhanced the ACTH response of the pituitary segments to CRH and increased the slope of its dose-response curve (Buckingham 1985). This potential effect is also confirmed *in vivo* (Fischman & Moldow 1984). The ovine fetal pituitary *in vivo* responds separately and synergistically to vasopressin and CRH (Norman & Challis 1987). The CRH-induced elevation of cAMP is potentiated (by an order of magnitude) by stimulators of PKC in a concentration dependent manner, which suggests that PKC, activated by V1b receptor stimulation, can modulate CRH receptor coupling to the adenylate cyclase (Cronin *et al.* 1986; Abou-Samra *et al.* 1987b). cAMP degradation is also decreased by inhibiting phosphodiesterase activity after PKC stimulation (Giguere & Labrie 1982; Abou-Samra *et al.* 1987b). Incubation of tissues with the phorbol ester phorbol myristate acetate (PMA), which depletes cell stores of PKC, selectively potentiated CRH-stimulated ACTH secretion and cAMP formation by anterior pituitary cells, but it failed to similarly affect AtT-20 cells exposed to CRH (Lutz-Bucher *et al.* 1990). However, AtT20 cells lack V1b receptor, and may lack the signalling mechanism that in corticotrophs "cross-talk" to the CRHR1 signalling pathway (Figure 1.1).

1.3.2.3.5 Regulation V1b receptor gene expression in the anterior pituitary and its functions

During chronic stress, there is a parallel relationship between V1b receptor content and responsiveness of the corticotrophs. Vasopressin binding is reduced after water deprivation and 2% saline administration where ACTH secretion is decreased, but it is increased after repeated intraperitoneal hypertonic saline injections which are painful, and repeated immobilisation where ACTH secretion is increased (Aguilera *et al.* 1994). V1b receptor mRNA levels are significantly increased after 8 and 14 days of repeated immobilisation or intraperitoneal hypertonic saline injection.

However, notably, changes in V1b receptor mRNA levels are not always correlated with changes in vasopressin receptor content. In response to acute stress, V1b receptor mRNA expression is increased after immobilisation, but is reduced 4 h after a single intraperitoneal hypertonic saline injection. While the content of V1b receptor in the pituitary increases after a single immobilisation, it is also increased after intraperitoneal hypertonic saline injection (Rabadan-Diehl *et al.* 1995). The decrease in V1b receptor mRNA expression following a single intraperitoneal hypertonic saline injection is prevented by pretreatment with a V1 receptor antagonist, suggesting that increased vasopressin secretion and other factors secreted into the hypophysial portal or peripheral circulation during this stress may account for the decrease of V1b receptor expression (Rabadan-Diehl *et al.* 1995). The high rate of mRNA utilisation or a transient transcriptional inhibition may account for the prolonged V1b receptor mRNA downregulation, while recruitment of receptors from intracellular pools or an increase in receptor synthesis at the posttranslational level may play a role in increasing vasopressin binding (Aguilera & Rabadan-Diehl 2000). The lack of parallelism between V1b receptor mRNA and vasopressin binding indicates that regulation of steady-state levels of V1b receptor mRNA is not a primary determinant in the control of pituitary vasopressin receptor concentration during stress.

Chronic injection of dexamethasone decreases vasopressin binding but increases V1b receptor mRNA expression (Rabadan-Diehl *et al.* 1997a; Rabadan-Diehl & Aguilera 1998). This suggests that glucocorticoids increase V1b receptor mRNA expression by stimulating gene transcription or/and by increasing mRNA stability, but decrease receptor number at the translational or posttranslational levels (Aguilera & Rabadan-Diehl 2000). Removal of circulating glucocorticoids by adrenalectomy transiently decreases pituitary V1b receptor mRNA levels by 18 h, which return to basal levels after 6 days, but results in sustained reduction in binding (Rabadan-Diehl *et al.* 1997a). The decrease in vasopressin binding could reflect a direct action of glucocorticoids on the pituitary (Lutz-Bucher *et al.* 1986) or an increase in receptor occupancy and receptor internalisation, because glucococorticoid deficiency results in increased concentrations of vasopressin and CRH in the pituitary portal blood due to removal of glucocorticoid negative feedback. However these increased concentrations of vasopression and CRH in the pituitary portal blood are unlikely to be the explanation for the reduced V1b receptor mRNA 18 h after adrenalectomy, because

PVN lesions or median eminence deafferentation does not prevent the transient V1b receptor mRNA downregulation (Rabadan-Diehl *et al.* 1997a).

In vitro studies demonstrate that activation of ACTH secretion by vasopressin is insensitive to feedback inhibition by glucocorticoids (Abou-Samra *et al.* 1986; Bilezikjian *et al.* 1987). While glucocorticoid administration results in pituitary vasopressin receptor loss (Koch & Lutz-Bucher 1985; Lutz-Bucher *et al.* 1986), so it is supposed that vasopressin receptor activity is enhanced by glucocorticoids. In the absence of glucocorticoids in the adrenalectomised rats, the loss of vasopressin receptor is accompanied by a blunting of the pituitary inositol-1,4,5 trisphosphate (IP_3) response to vasopressin; however, daily administration of dexamethasone (40 micrograms/day) reverses this effect of adrenalectomy (Todd & Lightman 1987). Rabadan-Diehl et al found that glucocorticoid treatment potentiated vasopressin stimulated IP_3 formation in cultured anterior pituitary cells and this potentiating effect of glucocorticoids is considered to result from facilitation of V1b receptor coupling to phospholipase C (Rabadan-Diehl & Aguilera 1998).

In summary, different stress types have different influence on V1b receptor expression, and it is not clear the roles of CRH and vasopressin in regulating this gene expression. Glucocorticoids increase V1b receptor mRNA expression and enhance V1b receptor activity in corticotrophs.

1.3.3 Oxytocin

The oxytocin precursor gene has similar exon-intron organisation to that of the vasopressin gene (Ivell & Richter 1984). Oxytocin is mainly synthesised in the magnocellular neurones in the PVN and SON of the hypothalamus (Rhodes *et al.* 1981), and minimally in the parvocellular neurones (Pretel & Piekut 1990). Oxytocin is transported through the internal layer of median eminence to the nerve terminals of the posterior pituitary, where it is released into the peripheral circulation. However, there are few oxytocin fibres detected in the external layer (Hoffman *et al.* 1989; Villar *et al.* 1994). Antoni et al. reported that a substantial amount of oxytocin in portal blood may be a consequence of preterminal release from supraoptic nuclei projections in the internal zone of the median eminence (Antoni *et al.* 1990).

Oxytocin secretion into pituitary portal plasma is increased by ovarian steroids, prolactin, and the suckling stimulus (Sarkar *et al.* 1992). Oxytocin secretion is also increased in rats in response to stress. Acute exposure of rats to immobilisation stress results in an increase in oxytocin mRNA level in magnocellular neurones (Jezova *et al.* 1995). Magnocellular neurones of the PVN, but not evidently the SON, are essential for oxytocin release during immobilisation stress (Jezova *et al.* 1995). The secretion of oxytocin in response to stress is inhibited by endogenous opioids (Douglas *et al.* 2000) and this inhibitory effect is greater in pregnant rats than in virgin rats (Douglas *et al.* 1998).

The functions of circulating oxytocin in uterine contraction during parturition and milk ejection during lactation are well established (Russell & Leng 1998). Oxytocin is also involved in the regulation of ACTH secretion from the anterior pituitary (Won *et al.* 1990). In the rats, oxytocin can potentiate the stimulatory effect of CRH in ACTH secretion *in vitro* (Gibbs *et al.* 1984). The injection of anti-oxytocin antiserum which completely neutralises the increase in plasma oxytocin levels during tail-hang stress causes a decrease in plasma ACTH concentration (Gibbs 1985). Brain oxytocin, in contrast to circulating oxytocin, inhibits basal and stress-induced HPA axis activity (Neumann *et al.* 2000b). In pregnancy, this brain effect on basal and stress-induced HPA axis activity is lost (Neumann *et al.* 2000a).

Receptors for oxytocin are present in the rat anterior pituitary (Antoni 1986b). However, oxytocin stimulates ACTH secretion via a receptor(s) other than the oxytocin receptor itself. Neither the oxytocin receptor agonist Thr^4Gly^7 oxytocin nor the oxytocin receptor antagonist des-$Gly(NH_2)^9d(CH_2)^5$-$[Tyr(Me)^2\ Thr^4]$OVT has any influence on basal ACTH release, and des-$Gly(NH_2)^9d(CH_2)^5$-$[Tyr(Me)^2\ Thr^4]$OVT does not interfere with oxytocin-induced ACTH release. A vasopressin receptor antagonist, dP$[Tyr(Me)^2]$vasopressin completely abolishes oxytocin-induced increases in ACTH release (Schlosser *et al.* 1994), suggesting that vasopressin V1b receptors mediate the actions of oxytocin on ACTH secretion.

1.4 Regulation of proopiomelanocortin (POMC) and ACTH synthesis

ACTH belongs to a family of peptides derived from a common precursor protein encoded by the proopiomelanocortin (POMC) gene (Chretien & Seidah 1981; King & Baertschi 1990). The POMC gene has been cloned and sequenced (Nakanishi *et al.* 1981; Cochet *et al.* 1982; Notake *et al.* 1983; Takahashi *et al.* 1983; Uhler & Herbert 1983; Drouin *et al.* 1985). The human POMC gene is about 11.6 kb and consists of three exons, in which the second and third exons encode the opiomelanocortin peptide (Cochet *et al.* 1982; Lundblad & Roberts 1988; King & Baertschi 1990). The upstream POMC gene DNA sequences have a glucocorticoid receptor-binding site, which probably mediates inhibition of the POMC gene transcription by glucocorticoid (Drouin *et al.* 1985). The POMC gene is strongly expressed in the anterior and intermediate pituitary gland (Civelli *et al.* 1982), and lesser amounts broadly distributed in the brain including the hypothalamus especially the arcuate nucleus, amygdala, cerebral cortex (Civelli *et al.* 1982), and in many peripheral tissues (Jingami *et al.* 1984; Pintar *et al.* 1984).

The POMC molecule is posttranslationally processed to different biologically active peptides. The cleavage of the POMC precursor is tissue specific: in the anterior pituitary corticotrophs, it is cleaved into ACTH (Figure 1.1), β-lipotropin and small amounts of β-endorphin (Lazarus *et al.* 1976; Lowry *et al.* 1980; Chretien & Seidah 1981; Lundblad & Roberts 1988; King & Baertschi 1990); in the intermediate lobe and other tissues, the processing continues to form α-melanocyte-stimulating hormone (α-MSH) and corticotropin-like intermediate lobe peptide from ACTH and γ-lipotropin and β-endorphin from β-lipotropin (Eipper & Mains 1980; Lundblad & Roberts 1988; King & Baertschi 1990). Opiomelanocortin peptides are stored within membrane-bound granules located mainly along the plasma membrane, and are released concomitantly in equimolar amounts upon stimulation with secretagogues (Moriarty & Halmi 1972; Allen *et al.* 1978; Vale *et al.* 1978; Westlund *et al.* 1985).

Stress increases POMC gene expression in corticotrophs. Intermittent electrical footshocks increase POMC mRNA levels in the anterior pituitary approximately 2-fold after three days treatment and this remains elevated for seven days (Hollt *et al.*

1986). Three weeks of chronic arthritis pain causes an 80% increase in anterior pituitary POMC mRNA levels (Millan *et al.* 1986). This stress-stimulated POMC expression may be due to the enhanced hypothalamic PVN CRH and vasopressin inputs. The treatment of primary anterior pituitary cells or AtT20 cells with CRH causes an increase in the levels of POMC mRNA. CRH exposure for eight hours stimulates POMC mRNA levels approximately 3-fold in AtT20 cells, an effect mimicked by the addition of 8-bromo-cAMP (a cAMP analogue) (Affolter & Reisine 1985). Both CRH and forskolin also increase the levels of POMC mRNA in primary anterior pituitary cell culture (Loeffler *et al.* 1985). *In vivo* studies show that PVN lesions decrease the levels of POMC mRNA in the anterior pituitary (Bruhn *et al.* 1984a), indicating that hypothalamic inputs during stress are responsible for the increased POMC expression. The potentiation of CRH- stimulated ACTH secretion by vasopressin is well established (Gillies *et al.* 1982). However, *in vitro* studies indicate that vasopressin does not potentiate CRH-induced accumulation of either cytoplasmic POMC mRNA (Suda *et al.* 1989c) or hnRNA (Levin *et al.* 1989).

POMC mRNA levels are regulated negatively by plasma glucocorticoid levels. Nakanishi et al first reported that adrenalectomy caused increases in POMC mRNA activity in a time dependent manner measured with the use of a cell-free protein-synthesising system, and in ACTH release in the anterior pituitary, which can be prevented by glucocorticoid replacement (Nakanishi *et al.* 1977). *In situ* hybridisation studies have found similar observations of adrenalectomy and dexamethasone or glucocorticoid replacement on pituitary POMC mRNA expression (Birnberg *et al.* 1983; Fremeau, Jr. *et al.* 1986). The treatment of primary anterior pituitary cells or AtT20 cells with either corticosterone or dexamethasone causes a decrease in the levels of POMC mRNA (Roberts *et al.* 1979; Eberwine & Roberts 1984).

1.5 Inhibitory factors of ACTH secretion

1.5.1 Glucocorticoid feedback

Glucocorticoids are synthesised in the zonae fasciculata/reticularis of the adrenal cortex and released into the circulation in response to a wide range of stressful

stimuli. Glucocorticoids have a vast array of functions within the body, including regulation of intermediary metabolism, and repression of immune responses. To maintain homeostasis, glucocorticoid secretion is also regulated by negative feedback. Negative feedback, or end-product inhibition, the most important homeostatic control mechanism of the HPA axis system is an autoregulatory feedback by glucocorticoids that can inhibit ACTH secretion both directly and indirectly.

1.5.1.1 Glucocorticoid metabolism

Upon entry through the cell membrane glucocorticoids encounter a major metabolising enzyme broadly distributed in the body, 11β-OH steroid dehydrogenase (11β-HSD), which inter-converts glucocorticoids and its inactive metabolites (Stewart *et al.* 1987; Funder *et al.* 1988; Krozowski *et al.* 1995). 11β-HSDs are responsible for glucocorticoid degradation. It comes in two isoforms, type 1 (11β-HSD1) and type 2 (11β-HSD2) with distinct and important roles. 11β-HSD2 almost irreversibly inactivates cortisol and corticosterone, oxidising their 11β-hydroxy group to the 11-keto forms, which are respectively, cortisone and 11-dehydrocorticosterone, and these bind only weakly to MRs and GRs. 11β-HSD1 catalyses both the oxidising (inactivating) and reducing (activating) reactions and so can reactivate the 11-keto steroids. The direction of the reaction depends on the presence of the co-factor, NADP(H), in the cells (Seckl & Walker 2001).

1.5.1.2 Types of glucocorticoid negative feedback

Corticosteroid action in the HPA axis system is classified on the basis of the time of onset of the biological actions on the release of ACTH by the anterior pituitary gland: rapid (within 30 min), delayed (1-2 h), and slow (more than 2 h) (Dayanithi & Antoni 1989; Nicholson & Gillham 1989). At the pituitary level feedback inhibition of ACTH secretion by adrenal glucocorticoids is exerted via type II, glucocorticoid receptors (GR), involving reduction of POMC synthesis. However, the breakdown of POMC mRNA, POMC protein and stored ACTH is not changed by glucocorticoid treatment (Phillips & Tashjian 1982; Roberts *et al.* 1987).

1.5.1.3 Mechanism(s) of glucocorticoid negative feedback

The mechanisms underlying rapid glucocorticoid inhibition of ACTH secretion from anterior pituitary corticotrophs are likely to involve the disruption of stimulus-secretion coupling independently of effects on ACTH biosynthesis, storage, or degradation because overwhelming evidence reveals that the predominant effect of glucocorticoids is to inhibit stimulated, rather than basal ACTH secretion (Clark & Kemppainen 1994; Shipston *et al.* 1996). Though some studies revealed inhibition of CRH-induced cAMP accumulation *in vitro* at the slow stage (Bilezikjian & Vale 1983; Bilezikjian *et al.* 1987), other studies show no change of CRH or stress-induced cAMP accumulation *in vivo* or *in vitro* in either the slow inhibition (Giguere *et al.* 1982; Kant *et al.* 1989) or rapid inhibition (Woods *et al.* 1992). Studies have shown that ACTH secretion stimulated by a direct activator of PKA or PKC is inhibited by glucocorticoids (Phillips & Tashjian 1982; Oki *et al.* 1991; Woods *et al.* 1992), but glucocorticoids do not prevent cAMP-induced activation of PKA (Miyazaki *et al.* 1984).

In vitro studies have shown that activation of ACTH secretion by vasopressin is less sensitive to feedback inhibition by glucocorticoids than that to CRH (Bilezikjian *et al.* 1987). *In vivo,* chronic dexamethasone treatment decreases the binding of [^{3}H]-vasopressin to pituitary membranes, in contrast, it increases V1b receptor mRNA and vasopressin-stimulated IP_3 formation as mentioned in Chapter 1.3.2.3.5 (Rabadan-Diehl *et al.* 1997a).

Glucocorticoids also inhibit Ca^{2+}-dependent ACTH secretion (Oki *et al.* 1991) by influencing the activation of either PKA or PKC, but not IP_3/Ca^{2+}-mediated ACTH release. Whole cell path clamp recordings indicate that glucocorticoids limit Ca^{2+} influx by enhancing a transient outward A-type K^+ current (Pennington *et al.* 1994). Patch-clamp studies show that the synthetic glucocorticoid dexamethasone prevents the inhibitory effect of PKA on BK-channels (Shipston *et al.* 1996).

In the PVN, Kovacs and her colleagues demonstrated that it is the vasopressin, and not the CRH, gene that is the principal target of glucocorticoid-mediated transcriptional suppression during stress (Kovacs *et al.* 2000).

1.5.1.4 Corticosteroid-binding globulin (CBG)

The circulating plasma level of corticosteroid-binding globulin (CBG), the major glucocorticoid transport protein, is an important factor regulating glucocorticoid availability to target tissues. Variations in plasma CBG levels will affect the free concentrations of glucocorticoids in the plasma and therefore influence the bioactive efficacy of the glucocorticoid signal. During the course of the normal diurnal cycle approximately 90% of corticosterone is bound to CBG in the circulation (Hammond *et al.* 1987). CBG belongs to the serine protease inhibitor family (Hammond *et al.* 1987). The majority of CBG is synthesised in the liver but CBG mRNA and protein have also been detected in rodents in the proximal tubules of the kidney (Scrocchi *et al.* 1993a). In mice and rats foetal hepatic CBG mRNA expression and plasma CBG concentrations are very high at mid-gestation and then drop to almost undetectable levels around birth (Smith & Hammond 1989; Scrocchi *et al.* 1993b). The anterior pituitary contains an intracellular CBG, transcortin, derived from plasma CBG (de Kloet & McEwen 1976; Koch *et al.* 1976). It exists in a membrane bound form as well as in the cytosol (Koch *et al.* 1978), where it functions much like plasma CBG in that it sequesters free corticosterone and decreases binding to GR in the anterior pituitary. Adrenalectomy increases, while chronic corticosterone treatment decreases plasma CBG levels (Feldman *et al.* 1979; Levin *et al.* 1987). Acute stress can decrease plasma and intracellular CBG levels 24 h after the end of the stress (Tannenbaum *et al.* 1997). Plasma CBG levels are also increased during pregnancy (Seal & Doe 1967; Smith & Hammond 1989).

1.5.1.5 Glucocorticoid receptor (GR)

The inhibitory effects of corticosterone on HPA axis function are believed to be transduced primarily by intracellular steroid receptors that function as hormone-activated transcription factors (Figure 1.1). Two different corticosteroid receptors have been cloned and sequenced: glucocorticoid receptor (GR, type II corticosteroid receptor) (Hollenberg *et al.* 1985) and mineralocorticoid receptor (MR, type I corticosteroid receptor) (Arriza *et al.* 1987). MR has a high affinity for both the mineralocorticoid, aldosterone, and the glucocorticoids, cortisol and corticosterone, and a lower affinity for the synthetic glucocorticoid dexamethasone (Beaumont & Fanestil 1983; Reul & de Kloet 1985; Sutanto & de Kloet 1987). GR has a high

affinity for dexamethasone, and a lower affinity for corticosterone and aldosterone (Veldhuis *et al.* 1982; Reul & de Kloet 1985).

GR cDNA has been cloned from rat (Miesfeld *et al.* 1984), mouse (Danielsen *et al.* 1986) and human (Hollenberg *et al.* 1985), and two protein forms of 777 (alpha) and 742 (beta) amino acids are predicted from hGR cDNA, differing at their carboxy terminal part with alternative splicing of exons 9α and 9β (Hollenberg *et al.* 1985). Both hGR isoforms share the same amino acid sequence through amino acid 727 but diverge beyond this position, hGRα with an additional 50 amino acids and hGRβ with an additional non-homologous 15 amino acids (Oakley *et al.* 1996). There are few studies about the function and distribution of hGRβ so far. hGRβ is largely ignored because it is not found to bind ligand or activate transcription of glucocorticoid-response reporter gene (Hollenberg *et al.* 1985; Giguere *et al.* 1986). From *in vitro* studies, hGRβ is considered as a dominant negative regulator of hGRα transactivation (Oakley *et al.* 1996; Oakley *et al.* 1997).

1.5.1.5.1 Distribution of MR and GR

Double immunohistochemical techniques and *in situ* hybridisation have revealed that GR mRNA is expressed in almost 99% of growth hormone cells and corticotrophs, and in 67% of thyroid stimulating hormone-producing cells. Almost all of the folliculostellate cells (93%), marginal layer cells (94%) in the anterior pituitary, and pituicytes (96%) in the posterior pituitary are colocolised with GR (Ozawa *et al.* 1999).

MRs are mainly present in the hippocampus and dorsolateral septum. The pattern of MR mRNA expression in the hippocampus is similar to that shown by *in vitro* autoradiography of MR binding, with highest levels in the CA1-CA2, pyramidal layer of CA3 and granular layer of the dentate gyrus (Van Eekelen *et al.* 1988; Herman *et al.* 1989).

GR immunoreactivities are present in the parvocellular part of the PVN, in the anterior periventricular hypothalamic nucleus, in the ventral part of the mediobasal hypothalamus, and in the CA1 and CA2 subregion of the hippocampal formation. Within the PVN a substantial overlap exists between the ir-GR area and the ir-CRH

area (Fuxe *et al.* 1985). Medium to high densities of GR immunoreactive neurones are present all over the cortical hemispheres, and in many thalamic nuclei and in the central amygdaloid nucleus (Fuxe *et al.* 1985). GR mRNA exists within the pyramidal neurones of the CA1 and CA2 areas of the hippocampal formation, in the granular cells of the dentate gyrus, in the parvocellular neurones of the PVN, and in the cells of the arcuate nucleus. It is also detected in a large number of neurones within layers II, III, and VI of the neocortex, in many thalamic nuclei, all over the granular layer of the cerebellar cortex, and the entire locus coeruleus and the mesencephalic raphe nuclei, rich in noradrenaline and 5-hydroxytryptamine cell bodies in the lower brainstem. There is a close correlation between the distribution of GR mRNA and the distribution of ir-GR (Aronsson *et al.* 1988; Van Eekelen *et al.* 1988; Yang *et al.* 1988).

1.5.1.5.2 Mechanism of glucocorticoid action via GR and MR

This dual receptor system for a single class of hormones is advantageous in dealing with the manifold physiological functions of glucocorticoids. Under resting conditions, plasma corticosterone levels undergo characteristic circadian fluctuations, ranging within 100 pg/ml. When homeostasis is disturbed by stressors, the corticosterone levels may fluctuate over a wide range (almost 50-fold) to several hundreds pg/ml. With two different type receptors, GR and MR, a sufficient flexible dynamic range is available. Reul and de Kloet have revealed that, in rats killed early in the morning (at the trough of the HPA axis diurnal rhythm; plasma corticosterone level <15 pg/ml), more than 80% MRs are already occupied by endogenous ligand, whereas GRs are only about 10% occupied (Reul & de Kloet 1985). In contrast, GRs become substantially occupied only under conditions of higher plasma corticosterone levels (>50-100 pg /ml) such as occur at the circadian peak and during stress (Reul & de Kloet 1985; Reul *et al.* 1987). So it is believed that GRs mediate the negative feedback of elevated glucocorticoid levels to restrain HPA drive, whereas MRs mediate the tonic inhibitory control of the hippocampus on HPA activity of basal activity (de Kloet & Reul 1987; Reul *et al.* 2000).

In the absence of ligand, the GR and MR are maintained in an inactive state, forming a heterooligomer with immunophilins (Tai *et al.* 1992), or with heat shock proteins (Pratt *et al.* 1988; Vamvakopoulos & Chrousos 1994). When glucocorticoids enter

target cells by passive diffusion, they bind to their cognate receptors (GR, MR) in the cytosol. However, there is some evidence (Harrison *et al.* 1979) suggesting that glucocorticoid entry into cells is a regulated process involving specific membrane-associated receptors distinct from the classical intracellular GRs. After intranuclear translocation, the GR-GR, MR-MR homodimers or the GR-MR heterodimer are constituted, depending on the relative levels of GR and MR and the concentrations of their ligands. The receptor dimers bind to glucocorticoid response elements (GREs) in the flanking region of target genes and regulate transcription of hormone-regulated genes (Trapp *et al.* 1994).

1.5.1.5.3 Regulation of GR expression in the anterior pituitary

In vivo studies have shown that adrenalectomy or CRH treatment alone does not change pituitary GR binding capacity (McEwen 1979) or GR mRNA (Sheppard *et al.* 1990), whereas CRH decreases and dexamethasone increases GR mRNA in the adrenalectomised rat pituitary (Sheppard *et al.* 1990), which suggests that the CRH-induced GR mRNA down-regulation is reversed by corticosterone *in vivo*. In the AtT20 cell line model, CRH treatment leads to a rapid time-dependent decrease in GR mRNA levels which precedes a dose- and time-dependent decrease in GR binding capacity. The relative proportion of nuclear *vs.* cytoplasmic localised [^{3}H]dexamethasone-bound GR does not differ between control and CRH-treated cultures, indicating that CRH does not interfere with GR nuclear translocation. Treatment with either forskolin or 8-bromo-cAMP, mimics the CRH-induced decrease in GR binding, and in addition forskolin decreases GR mRNA levels (Sheppard *et al.* 1991).

Sustained elevations of circulating corticosterone, whether by repeated stress or exogenous corticosterone administration, do not change receptor affinity for [^{3}H]dexamethasone, or receptor number in the pituitary (Sapolsky *et al.* 1984; Sapolsky & McEwen 1985). In contrast, equivalent doses of dexamethasone down-regulate pituitary corticosterone receptor numbers (Sapolsky & McEwen 1985). However, anterior pituitary GR mRNA expression increases after chronic glucocorticoid treatment (two 100 mg corticosterone pellets for 6 days, subcutaneous implant) (Sheppard *et al.* 1990). This discrepancy between GR binding assays and

mRNA studies may be explained in terms of translational efficiency and/or posttranslational modification.

Chronic treatment (5 days) with a small dose of dexamethasone (0.25 μg/ml), but not corticosterone (25 μg/ml), increases anterior pituitary GR mRNA levels in the adrenalectomised animals (Sheppard *et al.* 1990). These differences in the response to dexamethasone and corticosterone may partially be explained by the presence of steroid-binding proteins and/or modifying enzymes found in the pituitary (de Kloet *et al.* 1977; Koch *et al.* 1976). Both CBG and other CBG-like binding proteins would in effect lower the local concentration of corticosterone, but not dexamethasone, thus decreasing the ability of corticosterone to bind to corticosteroid receptors. The presence of modifying enzymes such as 11β-HSD is another factor that could decrease the local concentration of corticosterone, but not dexamethasone.

Sex steroids are also involved in GR gene expression regulation. Ovariectomy resulted in an approximately 2-fold increase in GR mRNA concentrations in the anterior pituitary. Administration of 17β-estradiol completely reverses the ovariectomy- induced increase in GR mRNA content of the pituitary gland (Peiffer & Barden 1987). Treatment of rats with corticosterone does not influence the ovariectomy-induced increase in glucocorticoid receptor mRNA content, indicating that this increase is not mediated via effects on circulating glucocorticoid levels or availability. GR receptor contents in the pituitary are also regulated by sex steroids. Gonadally intact females have less ^{3}H-dexamethasone binding than intact males. However, there is no difference in receptor concentration between ovariectomised females and gondadectomised males. Estrogen is able to reverse the effect of ovariectomy: ovariectomised females receiving estrogen have fewer receptors than intact males. Progesterone does not antagonise the effect of estrogen in the pituitary (Turner 1990).

1.5.2 Neurosteroids

The term "neurosteroid" was introduced by Baulieu in 1981 to describe a steroid hormone, dehydroepiandrosterone sulphate, that was found at high levels in the body

long after gonadectomy and adrenalectomy (Baulieu 1981). Androsterone, pregnenolone, tetrahydro- metabolites of progesterone, deoxycorticosterone (DOC), dehydroepiandrosterone, their sulphates, and lipid derivatives are identified as neurosteroids (Jung-Testas *et al.* 1989; Majewska 1992; Corpechot *et al.* 1993). Within this large family of neurosteroids, these hormones and their metabolites have a broad range of effects and the functions of these neurosteroids have been extensively studied. Allopregnanolone and 3α,21-dihydroxy-5α-pregnan-20-one (tetrahydrodeoxycorticosterone, THDOC) act as allosteric modulators of neurotransmitter receptors such as $GABA_A$, N-methyl-D-asparate (NMDA) and sigma receptors (Irwin *et al.* 1994; Monnet *et al.* 1995; Baulieu 1998). Neurosteroids also modulate glycine-activated chloride channels (Prince & Simmonds 1992), neural nicotinic acetylcholine receptors (Valera *et al.* 1992) and voltage-activated calcium channels (ffrench-Mullen *et al.* 1994), and consequently regulate electrical activity of neurones (Dubrovsky *et al.* 1982; Dubrovsky *et al.* 1985; Rupprecht 1997). Neurosteroids have been shown to modulate behaviour and mood, with anti-anxiety (Crawley *et al.* 1986), hypnotic (Mendelson *et al.* 1987), and anti-aggressive actions (Kavaliers 1988). They facilitate repair of myelin (Baulieu 1998; Morfin *et al.* 1992). Pregnenolone and its metabolites enhance memory in male mice, which suggests pregnenolone may facilitate the transcription of immediate early genes (Flood & Roberts 1988; Flood *et al.* 1992).

Neuroactive steroids are present in brain independently of peripheral sources (Robel & Baulieu 1995), so they can be produced by brain tissue (Jung-Testas *et al.* 1989; Papadopoulos *et al.* 1992; Melcangi *et al.* 1993). In the brain these neurosteroids are synthesised by oligodendrocytes and glial cells (Baulieu & Robel 1990; Melcangi *et al.* 1993). The majority of allopregnanolone and THDOC are derived from circulating progesterone, but they are also synthesised in the brain *de novo* from choleserol via pregnenolone (Corpechot *et al.* 1993; Cheney *et al.* 1995; Genazzani *et al.* 1998).

The brain expresses enzymes (3α- and 3β-hydroxysteroid oxidoreductase and 5α-steroid reductase) that catalyse the metabolism of pregnenolone and other steroid derivatives that are active at $GABA_A$ receptors (Kabbadj *et al.* 1993; Melcangi *et al.* 1993). Allopregnanolone is synthesised from progesterone by a two-step reaction. The

first step is irreversible and is conversion of progesterone to 5α-pregnanedione catalysed by a 5α-reductase. The second step is reversible, and is conversion of 5α-pregnanedione to allopregnanolone mediated by 3α-hydroxysteroid oxidoreductases. THDOC is converted from progesterone to deoxycorticosterone, then to THDOC catalysed by a 5α-reductase. 17β-N,N-diethylcarbomyl-4-aza-4-methyl-5α-androstan-3-one (4-MA) is a 5α-reductase inhibitor, which blocks allopregnanolone and THDOC production (Figure 1.4).

Pharmacological doses of neurosteroids, derivatives of progesterone such as allopregnanolone, also elicit sedative-hypnotic, anti-convulsant, and anti-conflict actions on mice and rats (Belelli *et al.* 1989; Bitran *et al.* 1991; Wieland *et al.* 1991; Kokate *et al.* 1994). These effects of 3α-hydroxysteroids have been related to their ability to facilitate GABA action at the $GABA_A$ receptor-coupled-Cl^- channels (Majewska *et al.* 1986). Allopregnanolone and THDOC are the most potent and efficacious endogenous compounds known that are able to enhance $GABA_A$ receptor function. Allopregnanolone is a high affinity, positive allosteric modulator of the $GABA_A$ receptor associated with a Cl- ionopore (Gee *et al.* 1988; Gee 1988; Peters *et al.* 1988; Turner *et al.* 1989; Puia *et al.* 1990). Allopregnanolone is active at nanomolar concentrations (Peters *et al.* 1988; Turner *et al.* 1989; Puia *et al.* 1990; Puia *et al.* 1993) and is 500-fold (Majewska 1992) more potent than progesterone at modulating the $GABA_A$ receptor.

Both brain extract and plasma neurosteroid concentrations are markedly increased shortly after application of different stress paradigms (Purdy *et al.* 1991; Korneyev *et al.* 1993; Barbaccia *et al.* 1994; Barbaccia *et al.* 1998). In particular, during the stress response brain content of allopregnanolone increases (Korneyev *et al.* 1993; Purdy *et al.* 1991) and may be significantly higher in some brain regions for instance in the olfactory bulb (Korneyev *et al.* 1993). These data indicate that neurosteroids such as allopregnanolone are part of the negative feedback mechanism in the stress response. Pretreatment of rats with a single dose of allopregnanolone significantly attenuates the elevation of plasma ACTH after emotional stress and attenuates the increase in vasopressin mRNA levels in the ventromedial subdivision of the hypothalamic PVN (Patchev *et al.* 1996). Allopregnanolone also inhibits basal acetylcholine release from

Figure 1.4 The metabolism pathway of progesterone

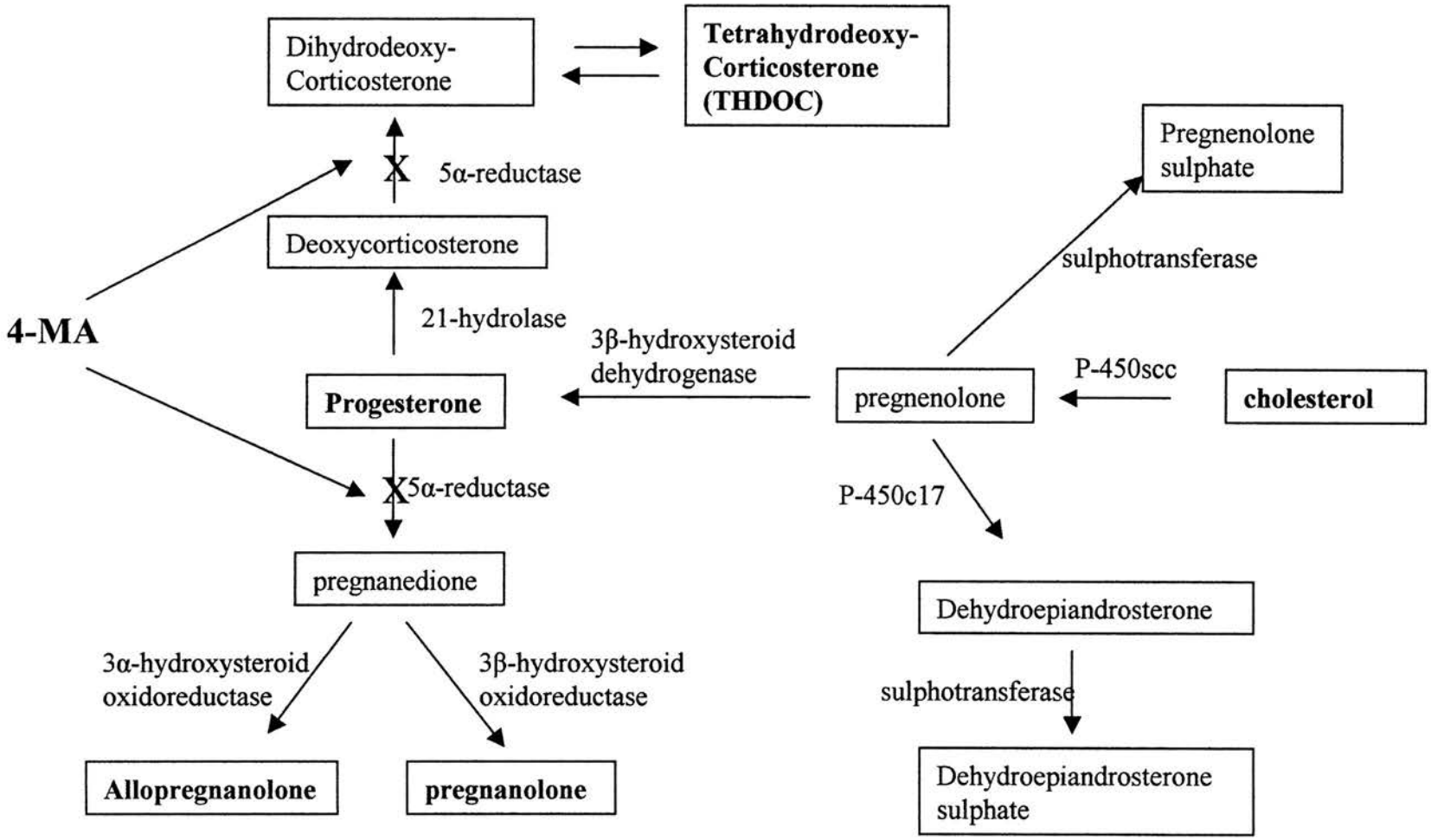


Figure 1.4 The metabolism pathway of progesterone (Phan, et al. 1999). 4-MA is an antagonist of 5α-reductase, blocking production of dihydrodeoxy-corticosterone from deoxycorticosterone, and pregnanedione from progesterone. As a result of 4-MA administration, allopregnanolone, THDOC and pregnanolone production are reduced.

the prefrontal cortex and hippocampus, in a dose-dependent manner. At a dose of 10 μg, allopregnanolone also completely prevents the increase in hippocampal acetylcholine release induced by foot-shock stress (Dazzi *et al.* 1996). Baseline plasma levels of ACTH are not affected by diazepam (an allosteric modifier at $GABA_A$ receptors), but both ether- and restraint-induced release of ACTH is reduced by diazepam (Bernet *et al.* 2000). Bilateral microinjection of muscimol (a $GABA_A$ receptor agonist) into either the PVN prior to air stress reduces the associated increases in plasma ACTH (Stotz-Potter *et al.* 1996). Intracerebroventricular injection of 10 μl of anti-allopregnanolone serum significantly potentiates the plasma corticosterone response to an acute cold water swimming stress (Guo *et al.* 1995). THDOC (5 mg/kg) attenuates mild stress- induced increases in plasma corticosterone concentrations via GABAergic mechanisms (Owens *et al.* 1992).

The release of CRH from the hypothalamus is decreased by activation of $GABA_A$ receptors, therefore, these neurosteroids may inhibit CRH release and subsequent ACTH release via $GABA_A$ receptor sites in the hypothalamus (Gram & Christensen 1986; Calogero *et al.* 1988b; Calogero *et al.* 1988a; Owens *et al.* 1992). A number of experimental studies clearly suggest that benzodiazepines attenuate CRH secretion possibly through inhibitory GABAergic mechanisms in human. The administration of alprazolam leads to a highly significant attenuation of the ACTH and cortisol increase following the "stress interview". Following administration of CRH, the ACTH augmentation is only slightly affected following alprazolam, while there are no changes in cortisol or prolactin secretion (Rohrer *et al.* 1994).

A vast majority of CRH neurones (>94.5%) are found to express transcripts for α1, β1 and β2 subunits of the $GABA_A$ receptor, and mRNAs for α1 and β2 subunits of the $GABA_A$ receptor are detected within 53.3% and 65.7% of PVN CRH neurones, respectively (Cullinan 2000). The subunit composition of $GABA_A$ receptors at this key regulatory locus may affect the efficacy of a major inhibitory input, and thus the magnitude and/or duration of stress-induced glucocorticoid secretion. Measurements of the steady-state levels of CRH mRNA in the PVN by quantitative *in situ* hybridisation shows that allopregnanolone is as potent as corticosterone in preventing adrenalectomy-induced up-regulation CRH mRNA expression (Patchev *et al.* 1994). Allopregnanolone does not alter the basal release of CRH from the hypothalamus *in*

vitro, but suppresses the stimulatory effect on CRH release by the α1-adrenergic agonist methoxamine (Patchev *et al.* 1994).

In vitro studies show that the neurosteroids dehydroepiandrosterone, pregnenolone and pregnanolone do not affect POMC mRNA levels on pituitary AtT 20 cells, even at the highest concentrations employed, suggesting that neurosteroids do not control POMC gene expression at the level of the pituitary corticotroph (Vedder *et al.* 1993).

In cortical neurones allopregnanolone positively modulates GABA elicited Cl^- currents (Puia *et al.* 1993). This neurosteroid has also been found to protect against seizures that arise from blockade of the GABA- Cl^- channel complex. Allopregnanolone induced a significant increase in $^{36}Cl^-$ uptake within 10 s compared with that induced by exposure to 100 μM GABA (Brinton 1994).

In summary, the metabolites of progesterone, allopregnanolone and THDOC, exert inhibitory effects on the HPA system, by inhibiting CRH and vasopressin production, but it is unlikely these neurosteroids have direct action on corticotrophs.

1.6 Other ACTH secretion regulation factors

Besides the factors discussed above, other stimulatory factors (review, (King & Baertschi 1990)) such as angiotensin II, vasoactive intestinal peptide, and catecholamines, and inhibitory factors (review, (Jessop 1999)) such as opioids, leptin, lipocortin, somatostatin, prepro-thyrotrophin- releasing hormone, substance P, nitric oxide, endothelins and adrenomedullin are also involved in regulating ACTH secretion from corticotrophs. CRH binding protein (CRHBP) is known to inhibit ACTH secretion by binding to CRH (Suda *et al.* 1988a). Atrial natriuretic peptide (ANP) is considered as an inhibitory factor of ACTH secretion (Kovacs & Antoni 1990), however, its role in regulating ACTH secretion is still controversial.

1.6.1 CRH binding protein (CRHBP)

CRHBP is a protein that binds CRH to modulate ACTH secretion from corticotrophs (Figure 1.1), but is not a receptor, and is found in the anterior pituitary, brain and other tissues. In particular, it is secreted into blood from placenta during human (but not rat) pregnancy.

In the late 1980's, CRHBP was found in both normal human plasma (Orth & Mount 1987; Potter *et al.* 1991) and late gestational maternal plasma (Perkins *et al.* 1995b). In addition to human placenta, CRHBP is also secreted in the intrauterine tissues (maternal decidua, amnion, and chorion) (Petraglia *et al.* 1993) and peritoneal fluid (Petraglia *et al.* 1997), but not in human ovarian follicles (Asakura *et al.* 1997).

1.6.1.1 Characterisation of CRHBP

Human CRHBP, a 37-38 Kd protein, is a glycoprotein that contains asparagine N-linked-type oligosaccharides, and such oligosaccharide chains are important for CRHBP binding (Suda *et al.* 1988a; Behan DP *et al.* 1989; Suda *et al.* 1989b). Human CRHBP binds specifically to CRH, but it is unlikely that CRHBP is a CRH receptor, because the estimated molecular weight of the CRHBP is smaller than the reported size of CRH receptors (anterior pituitary: 75 Kd; brain: 58 Kd (Grigoriadis & De Souza 1989b; Grigoriadis & De Souza 1989a)), and CRHBP has low affinity for the ovine CRH (Suda *et al.* 1988a; Sutton *et al.* 1995). The rank orders of affinity of the CRH family members for human CRHBP are: carp urotensin >> human CRH = rat/ovine urocortin > human urocortin >> frog sauvagine >> ovine CRH (Baigent & Lowry 2000b; Behan *et al.* 1996c).

CRHBP cDNA has been isolated (Behan DP *et al.* 1989) and cloned from rat (Potter *et al.* 1991), mouse (Cortright *et al.* 1995), sheep (Behan *et al.* 1996a; Cortright *et al.* 1997), and Xenopus (Valverde *et al.* 2001). The rat and human CRHBP gene spans almost 12000 bp and contains 7 exons and 6 introns encoding 322 amino acids with 5 tandem disulphide bridges which are essential for the binding of CRH (Behan *et al.* 1995; Behan *et al.* 1996b; Cortright *et al.* 1997; Zhao *et al.* 1997).

In primates the CRHBP gene is expressed in the anterior pituitary, brain, liver and placenta, whereas in rodents it is produced only in the brain and pituitary (Behan *et al.* 1995).

1.6.1.2 CRHBP localisation in the anterior pituitary and brain

In the anterior pituitary, CRHBP is expressed predominantly and extensively in corticotrophs and colocalises with ir-ACTH in a majority of corticotrophs (Potter *et al.* 1992).

CRHBP expression in the hypothalamus appears largely limited to the ventral premammillary and dorsomedial nuclei; only isolated CRHBP-stained cells are apparent in neurosecretory cell groups. Dual immunostaining for CRH and CRHBP reveals a partial colocalisation in some of these regions. Prominent CRHBP-stained terminal fields have been identified in association with CRH-expression cell groups in circumscribed hypothalamic and limbic structures. However, the PVN and median eminence, the hypothalamic sites of CRH synthesis and secretion, respectively, have only sparsely scattered ir-CRHBP fibres (Potter *et al.* 1992).

CRHBP is also expressed predominantly in the cerebral cortex, subcortical limbic system structures (amygdala, bed nucleus of the stria terminalis). Within the cerebral cortex and the hippocampal formation, the colocalisation of the CRH- and CRHBP-immunoreactivities is only in relative small subsets of neurones at a limited number of sites. More extensive instances of CRHBP and CRH peptide colocalisation are in the olfactory bulb, the ventral part of the lateral septal nucleus, the bed nucleus of the stria terminalis, the medial preoptic area, the central nucleus of the amygdala, the mesencephalic central gray, the interpeduncular nucleus, the lateral dorsal tegmental nucleus, the inferior colliculus, and the lateral reticular nucleus (Potter *et al.* 1992).

In the brain, CRHBP is associated with the intracellular membrane(s) (Behan *et al.* 1995). In the anterior pituitary ir-CRHBP is distributed diffusely throughout the cytoplasm of corticotrophs, but is associated with membrane-bound vesicular and vacuolar structures and in particular lysosomes and multivesicular bodies. Labelling for ir-CRHBP in the anterior pituitary is most prominently associated with the lysosomal system. These display either an otherwise homogeneous and finely

granular content (primary lysosomes) and are often seen in association with the Golgi apparatus or larger granules and/or vesicular structures (secondary lysosomes) distributed more widely in the cytoplasm. There is no association of ir-CRHBP with secretory granules (Peto *et al.* 1999).

There is no CRHBP in secretory granules in the pituitary cells, suggesting that it is not co-secreted with ACTH (Peto *et al.* 1999). This is consistent with no report of basal or stimulated CRHBP secretion from the anterior pituitary. The predominant association of the ir-CRHBP with secondary lysosomes, multivesicular bodies, and endosome-like vesicular structures in corticotroph cells suggests a principle role for the CRHBP in the processing and/or degradation of CRH or of ligand-receptor complex. CRHBP is in a position to participate in the signal transduction of the CRH/receptor complex. The roles of CRHBP are considered to be facilitating dissociation of the complex, with consequent freeing of the receptor for recycling to the cell surface, and/or acting as chaperone to direct internalised CRH to the lysosomal system of digestion (Peto *et al.* 1999).

1.6.1.3 Physiological functions of CRHBP

In primates, plasma CRHBP levels increase during pregnancy which completely abolish the ACTH releasing activity of CRH by binding with CRH forming a stable dimer to keep the normal ACTH response to stress (Linton *et al.* 1990; Woods *et al.* 1994b).

CRHBP inhibits CRH-induced ACTH secretion from cultured rat anterior pituitary cells at concentrations commonly found in late gestational maternal plasma (1.5 and 100 ng/ml, human CRH and CRHBP respectively) (Behan DP *et al.* 1989; Linton *et al.* 1990). In transgenic mice with CRHBP deficiency, the basal ACTH and corticosterone concentrations and the pattern of increased corticosterone and ACTH after restraint stress do not change (Karolyi *et al.* 1999). However, in the transgenic mice expressing elevated levels of CRHBP, a significant attenuation of ACTH secretion was observed at 3 h after lipopolysaccharide injection (Lovejoy *et al.* 1998). These data suggest that HPA axis regulation is significantly affected only with very high circulating levels of CRHBP. However, another model of transgenic study found the transgenic mice expressing elevated levels of CRHBP show similar ACTH

response to stress compared with normal mice (Burrows *et al.* 1998). The stress-induced CRH release into the hypothalamic portal system may be not quenched completely by the excess CRHBP, because the concentration of CRH released is high, and the exposure time before reaching the pituitary corticotrophs is low. As CRH and vasopressin mRNA levels increase by 82 and 35% respectively in the PVN of transgenic mice expressing elevated levels of CRHBP compared with the controls, respectively, this suggests increased CRH and vasopressin release compensates for the excess CRHBP (Burrows *et al.* 1998).

Since long term secretion of placental CRH into the peripheral circulation occurs during the third trimester of human pregnancy, the presence of circulating CRHBP may explain how markedly elevated plasma levels of CRH coexist with normal ACTH levels at this time (Suda *et al.* 1988a; Suda *et al.* 1989a).

1.6.1.4 CRHBP gene expression regulation

The mechanism of CRHBP gene expression regulation is not clear, however, there are some studies which suggest that CRHBP mRNA expression in the anterior pituitary is positively regulated by cAMP, CRH, stress and glucocorticoids (Cortright *et al.* 1997; McClennen *et al.* 1998). cAMP response element (CRE)-binding sites have been identified in the CRHBP promoter sequence and CRHBP promoter activity is positively regulated by cAMP and CRH via these CRE-binding sites (Cortright *et al.* 1997). In AtT20 cells, CRH positively regulates CRHBP promoter activity via the CRH receptor (Cortright *et al.* 1997). Forskolin, CRH, interleukin 6, and dexamethasone increased CRHBP mRNA and protein expression in dissociated fetal amygdalar cultures (Kasckow *et al.* 1999), whereas forskolin increases CRHBP mRNA levels, but dexamethasone can repress forskolin- induced CRHBP hnRNA levels in primary rat astrocyte cultures (McClennen & Seasholtz 1999). The mechanism of regulation of CRHBP gene expression by glucocorticoids is not clear. Two hours after restraint stress, CRHBP gene expression in the anterior pituitary increases two-fold over the basal level. Adrenalectomy decreased CRHBP mRNA expression level to 8% of the control level, but 30 min restraint stress may further increase CRHBP gene expression in the adrenalectomised rats (McClennen *et al.* 1998). That the ir-CRHBP concentration in human plasma is regulated by glucocorticoids is also suggested by clinical studies. However, the direction of control

of circulating CRHBP is opposite to that of control of expression in the pituitary and brain from the above studies. The CRHBP concentration is low in patients with Cushing's syndrome, after surgical treatment, the plasma CRHBP concentration in patients with Cushing's syndrome rose, peaked, and then decreased to the control level. In patients with Addison's disease, the high plasma CRHBP concentration decreased to the control level after hydrocortisone replacement (Ohmori *et al.* 1994; Suda *et al.* 1990).

1.6.2 Atrial natriuretic peptide (ANP)

ANP is generally known to be important in maintaining electrolyte and fluid homeostasis through inhibition of aldosterone and vasopressin release (Sonnenberg *et al.* 1982; de Bold *et al.* 2001). ANP was first reported to attenuate basal ACTH secretion from normal rat anterior pituitary by Shibasaki et al. (Shibasaki *et al.* 1986). Evidence from *in vivo* studies (Kovacs & Antoni 1990; Fink *et al.* 1991; Antoni *et al.* 1992; Fink *et al.* 1992) also suggests that ANP may be an ACTH release inhibitor. Ir-ANP is detected in some hypothalamic neurones, in the external zone of the median eminence and in the portal blood (Skofitsch *et al.* 1985; Kawata *et al.* 1985; Standaert *et al.* 1986; Sheward *et al.* 1991). ANP receptors are also found in the anterior pituitary (Koch *et al.* 1988). However, its role in regulating ACTH secretion is still controversial. Many studies have not found this inhibitory effect on ACTH secretion *in vivo* or *in vitro* (Heisler *et al.* 1986; Abou-Samra *et al.* 1987a; Hashimoto *et al.* 1987; Bowman *et al.* 1997; Mulligan *et al.* 1997).

1.7 HPA function during pregnancy

1.7.1 Changes in HPA axis activity during pregnancy

Adrenal glucocorticoids play a crucial role in overall metabolic homeostasis and allow the organism to respond adequately to various stressful stimuli. The adaptation of this axis may be crucial to pregnancy maintenance, since this condition arguably imposes the most dramatic and prolonged physiological stress encountered in normal postnatal life.

During pregnancy, the HPA axis respond to physical and emotional stress are attenuated (Neumann *et al.* 1998). The effect of exogenous CRH on the ACTH and corticosterone secretion is also reduced (Neumann *et al.* 1998).

Blood corticosterone levels were reported to fall, or not to change in early pregnancy, followed by a return to pre-pregnancy levels and a further increase at the latest stage of pregnancy in rats (Garland *et al.* 1987; Waddell & Atkinson 1994), mice (Montano *et al.* 1991) and humans (Nolten & Rueckert 1981). This increase in blood concentration may be attributed to either increased corticosterone production and/or reduced corticosterone clearance rate (Waddell & Atkinson 1994) in late pregnancy. Secondary endocrine and metabolic changes associated with pregnancy, i.e. increased ovarian oestrogen secretion which occurs in rat pregnancy could also increase adrenal corticosterone secretion by increasing sensitivity to ACTH. Corticosterone secreted by the foetal adrenal glands can also make a contribution (Dupont *et al.* 1991).

Glucocorticoid levels are controlled by ACTH. There is no study to investigate the clearance rate of ACTH during pregnancy so far, but it is reported that ACTH clearance rate is not altered by sex steroids (Viau & Meaney 1991). Increased CBG levels during pregnancy may reduce corticosterone clearance (Lin *et al.* 1990; Scott *et al.* 1990), and in pregnancy, the sensitivity of the adrenal gland to ACTH is increased (Waddell & Atkinson 1994). At the late stage of pregnancy, plasma corticosterone concentrations are slightly increased, but morning basal plasma ACTH concentrations do not change (Neumann *et al.* 1998). This also suggests that the sensitivity of the adrenal cortex to ACTH is increased during pregnancy.

Although basal morning plasma ACTH concentrations do not change during pregnancy, however there is a loss of the diurnal increase (Atkinson & Waddell 1995). The maternal placenta may be another source of circulating ACTH in the rat (Chen *et al.* 1986), but the placenta does not release ACTH in response to maternal stress (Ohkawa *et al.* 1991).

1.7.2 Possible mechanisms of the attenuated HPA axis activity in pregnancy

The mechanisms of this attenuated HPA axis response are not clear.

There are changes in the brain including the hypothalamus, hippocampus and other brain regions. Lower basal CRH and vasopressin mRNA expression in the PVN of pregnant rats suggests reduced CRH and vasopressin availability for driving the secretion of ACTH (Johnstone *et al.* 2000).

The sensitivity of corticotrophs during pregnancy may be changed as suggested from receptor autoradiography studies: CRH and vasopressin receptor binding in the anterior pituitary is decreased in pregnant rats compared with virgins (Toufexis *et al.* 1999; Neumann *et al.* 1998). cAMP levels in pituitary segments also show a pattern of reduced basal level, and a lower increase after CRH stimulation in pregnant rats (Neumann *et al.* 1998).

Glucocorticoid feedback may be involved in this change (Johnstone *et al.* 2000). GR mRNA expression measured by *in situ* hybridisation in the dentate gyrus is reduced, but not in the PVN, and 11β-HSD1 activity is also increased in the PVN and anterior pituitary at the late stage of pregnancy. This increase in the reductase, 11β-HSD1, may enhance the local intracellular glucocorticoid levels to exert negative feedback in the HPA axis.

The changes in sex steroid concentration may be another cause of the attenuated HPA axis activity. The effects of a subcutaneous implantation of progesterone and estradiol in ovariectomised rats suggests that estradiol facilitates HPA activity while progesterone inhibits such facilitatory effects (Carey *et al.* 1995). However, another study shows HPA activity is suppressed by chronic low-level estrogen replacement (Dayas *et al.* 2000). The inhibitory effect of progesterone may be through its metabolites such as allopregnanolone (see Chapter 1.5.2). The concentration in the brain of neuroactive steroids, allopregnanolone and THDOC (the metabolites of progesterone) are also at peak values prior to parturition (Concas *et al.* 1999). These neurosteroids exert an important inhibitory effect on the HPA system by interacting with the $GABA_A$ receptor in brain (Chapter 1.5.2) (Breier *et al.* 1992; Owens *et al.*

1992; Patchev *et al.* 1994; Patchev *et al.* 1996; Bernet *et al.* 2000; Cullinan 2000). So these neurosteroids may exert important role in the attenuated HPA axis responses to stress during pregnancy.

In the late 1980's, CRHBP was found in both normal human plasma (Orth & Mount 1987; Potter *et al.* 1991) and late gestational maternal plasma (Perkins *et al.* 1995b). During human pregnancy, maternal plasma ir-CRH level increases progressively. A significant amount of placenta CRH mRNA is detected and CRH protein is secreted by the placenta (Shibasaki *et al.* 1982; Grino *et al.* 1987), indicating that the placenta is a site of CRH biosynthesis during pregnancy. The secretion by the placenta results in large concentrations of CRH appearing in the maternal blood during the third trimester of pregnancy, reaching levels just before parturition similar to those found in the hypothalamic portal blood during stress in experimental animals (Campbell *et al.* 1987). However plasma ACTH concentrations during human pregnancy remain within the normal physiological range (Rees *et al.* 1975), and there is no correlation between plasma cortisol and CRH (Campbell *et al.* 1987). The excessive plasma CRH in pregnant women is bound to CRHBP so is inactive, therefore plasma ACTH levels do not increase to above the normal range (Suda *et al.* 1988a; Suda *et al.* 1989a). The rodent placenta does not produce significant amounts of CRH, therefore, a binding protein would not be required to protect the pituitary (Baigent & Lowry 2000a). However, CRHBP is expressed predominantly and extensively in corticotrophs and colocalises with ir-ACTH in a majority of corticotrophs in the anterior pituitary (Potter *et al.* 1992), and CRHBP is distributed broadly in the brain, so regulation of the HPA axis activity by CRHBP during pregnancy may change as well.

At the level of the anterior pituitary, the attenuated ACTH response to stress can be due to two possibilities: changes in the production of stimulatory and inhibitory factors from the brain or periphery, and changes in sensitivity of the corticotrophs to these factors. In this thesis, we planned to investigate mainly changes in CRH and vasopressin inputs from the hypothalamus and the cellular signalling changes in corticotrophs.

Aims of the thesis

Previous studies show that the attenuated HPA axis activity in pregnancy may be due to reduced sensitivity of corticotrophs to secretagogues and reduced CRH and vasopressin secretion from the hypothalamus.

The aims of this thesis were: 1) to determine whether changes in the anterior pituitary corticotrophs underlie reduced stress responses in pregnancy. By *in situ* hybridisation, we measured the expression of POMC, CRHR1, V1b receptor, GR, BK channel, and CRHBP mRNAs.

2) to determine whether ACTH secretion by anterior pituitary cells regulated by cAMP, CRH and vasopressin *in vitro* is different in pregnancy.

3) to elucidate the roles of CRH and vasopressin in ACTH secretion in pregnancy. We measured effects of CRH and vasopressin antagonists on ACTH responses to stress, and of exogenous vasopressin, combined with CRH on ACTH secretion.

4) to investigate the role of progesterone metabolites on the HPA axis activity. We blocked the production of allopregnanolone and THDOC by administering 4-MA and measured ACTH concentrations in response to swimming stress.

The thesis is about testing the two hypotheses underlying the attenuated ACTH response to stress during pregnancy: decreased sensitivity of the corticotrophs and/or reduced production of CRH and vasopressin by the hypothalamus.

Chapter 2

Materials and methods

Chemicals

8-CPT-cAMP	Biolog Life Science Institute, Germany
17β-N,N-Diethylcarbamoyl-4-methyl-4-aza- 5α-androstan-3-one (4-MA)	A gift from Prof. Ian Mason, Edinburgh, UK
Acetic anhydride	BDH (Merck Ltd.), UK
Acetone	BDH (Merck Ltd.), UK
Adrenocorticotropin (ACTH)	Bachem AG, Switzerland
ACTH Antibody	A gift from Prof. P.J.Lowry
Agarose	Promega UK Ltd., UK
Amberlite resin	BDH (Merck Ltd.), UK
Ammonium acetate	Sigma-Aldrich Company Ltd., UK
Ammonium persulphate	Sigma-Aldrich Company Ltd., UK
Ampicillin	Sigma-Aldrich Company Ltd., UK
Antalarmin	Gift from Dr. Manolis Zoumakis, NIH, USA
Anti-sheep IgG (for IRA)	Scottish Antibody Production Unit
Aprotinin	Sigma-Aldrich Company Ltd., UK
(Arg^8)- vasopressin	Bachem AG, Switzerland
Boric acid	Sigma-Aldrich Company Ltd., UK
Bovine serum albumin (BSA)	Sigma-Aldrich Company Ltd., UK
Calcium chloride	BDH (Merck Ltd.), UK
Chloroform/isoamyl (24:1)	Sigma-Aldrich Company Ltd., UK
Citric acid	Sigma-Aldrich Company Ltd., UK

Corticotropin releasing hormone (CRH)	Bachem AG, Switzerland
Cremophor EL	Sigma-Aldrich Company Ltd., UK
Denhardt's 50x	Sigma-Aldrich Company Ltd., UK
Dextran sulphate	Sigma-Aldrich Company Ltd., UK
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich Company Ltd., UK
Dipotassium hydrogen orthophosphate anhydrous (K_2HPO_4)	BDH (Merck Ltd.), UK
Disodium hydrogen orthophosphate (Na_2HPO_4)	Sigma-Aldrich Company Ltd., UK
Dithiothreitol (DTT)	Sigma-Aldrich Company Ltd., UK
Dulbecco's Modified Eagle's Medium (DMEM)	Life Technologies, UK
DNAse I	Sigma-Aldrich Company Ltd., UK
EDTA (disodium dihydrate)	Sigma-Aldrich Company Ltd., UK
Ethidium Bromide	BDH (Merck Ltd.), UK
Formamide	BDH (Merck Ltd.), UK
Formaldehyde	BDH (Merck Ltd.), UK
Gelatin	BDH (Merck Ltd.), UK
Halothane	Mackenzie& Co. Ltd., UK
Hydrochloric acid (HCl)	BDH (Merck Ltd.), UK
Isopropanol	BDH (Merck Ltd.), UK
Lima bean trypsin inhibitor	Sigma-Aldrich Company Ltd., UK
Multiparin (Heparin)	CP Pharmaceuticals Ltd, UK
Nick column, sephadex G50	Amersham Pharmacia Biotech, UK
Nifedipine	Sigma-Aldrich Company Ltd., UK
Nonimmune sheep serum matched for Anti-sheep IgG	Scottish Antibody Production Unit
Oligonucleotides (POMC, BK., ST)	MWG AG Biotech, Germany

Paraformaldehyde	Sigma-Aldrich Company Ltd., UK
Phenol/chloroform	Sigma-Aldrich Company Ltd., UK
Poly (A) [adenyl acid]	Sigma-Aldrich Company Ltd., UK
Polyethylene-glycol-6000	BDH (Merck Ltd.), UK
Poly-L-lysine coated slide	BDH (Merck Ltd.), UK
Polymerase	Promega, UK
Potassium chloride (KCl)	BDH (Merck Ltd.), UK
Potassium dihydrogen orthophosphate (KH_2PO4)	BDH (Merck Ltd.), UK
QIAquick nucleotide removal kit	QIAgen, Germany
QIAGEN plasmid midi kits	QIAgen, Germany
RNase A	Sigma-Aldrich Company Ltd., UK
Restriction endonucleases and reaction buffers (all types)	Promega, UK
Salmon Testis DNA	Sigma-Aldrich Company Ltd., UK
SequGel	National Diagnostics, UK
Sodium acetate	BDH (Merck Ltd.), UK
Sodium azide	BDH (Merck Ltd.), UK
Sodium bicarbonate (Na_2CO_3)	BDH (Merck Ltd.), UK
Sodium chloride (NaCl)	BDH (Merck Ltd.), UK
Sodium citrate	Sigma-Aldrich Company Ltd., UK
Sodium dihydrogen orthoposphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	Sigma-Aldrich Company Ltd., UK
Sodium dihydrogen orthoposphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	Sigma-Aldrich Company Ltd., UK
Sodium hydroxide (pellets)	BDH (Merck Ltd.), UK
Sodium pyrophosphate (PPI)	Sigma-Aldrich Company Ltd., UK
Temed	Sigma-Aldrich Company Ltd., UK
Terminal transferase kit	Biehringer Mannheim, Germany

Total RNA	Sigma-Aldrich Company Ltd., UK
Trascription buffer	Promega, UK
Triethanolamine	Sigma-Aldrich Company Ltd., UK
Triton-100	BDH (Merck Ltd.), UK
Trypsin TRL	Worthington Biochemical Corporation. USA
V1a/b receptor antagonist	Gift from Prof. Maurice Manning, Medical College of Ohio, USA
Xylene	BDH (Merck Ltd.), UK
Yeast extract	Sigma-Aldrich Company Ltd., UK
Yeast total RNA	Sigma-Aldrich Company Ltd., UK
Yeast tRNA	Sigma-Aldrich Company Ltd., UK

Radiochemicals

C^{14} polymer strip standard slides	Amersham Pharmacia Biotech, UK
^{35}S-ATP	NEN Life Science Products, Inc., USA
^{35}S-UTP	NEN Life Science Products, Inc., USA
^{125}I-ACTH	Phoenix Pharmaceuticals Inc., USA
^{125}I-ACTH kits	ICN Biomedicals, Inc., USA

Miscellanous

Coverslips	BDH (Merck Ltd.), UK
D-19 developer	Ilford Ltd., UK
DPX mountant	BDH (Merck Ltd.), UK
Halothane	Merial Animal health Ltd., UK
Hyperfilm β-max	Kodak
Ilford Hypam rapid fixer	Ilford Ltd., UK
Lens cleaning tissue	Whatman International Ltd.
Super premium microscope slides	BDH (Merck Ltd.), UK
Parafilm	AZWELL Inc., Japan

Polylysine coated microscope slides	BDH (Merck Ltd.), UK
PVC tubing	Altec Products Ltd, UK
Sesame oil	Sigma-Aldrich Company Ltd., UK
Silastic tubing	Altec Products Ltd, UK

2.1 *In vivo* studies

2.1.1 Animal maintenance

Adult female Sprague-Dawley rats (230-250 g) and female outbred MF1 mice (obtained from Bantin and Kingman, UK), were used for all *in vivo* and *in vitro* studies. The rats were caged in groups of 3-5 (to minimise stress-induced activation of the HPA axis) under standard laboratory conditions (lights on 7:00 h, lights off 19:00 h; 21°C with food and tap water *ad libitum*) and allowed to acclimatise in the MFAA unit, Edinburgh University for a period of at least two weeks before experiments or being mated. The animals were handled daily for a minimum of a week prior to an experiment in order to reduce non-specific stress effects occurring during experimentation.

To obtain pregnant rats, adult female rats or mice were caged individually with a sexually experienced male in mesh floored breeder cages. If a vaginal plug of semen appeared on the underlying tray the next morning then that day was designated as day 1 of pregnancy. After mating, rats or mice were housed separately until the day of experiment. The number of pups was counted *post mortem* and data were excluded if less than four pups were found.

2.1.2 Surgery

2.1.2.1 Chronic jugular cannulation

Under halothane: nitrous oxide anaesthesia pregnant and virgin rats were implanted with a jugular cannula for blood sampling. Using sterile procedures the right jugular vein was carefully exposed through a small skin incision and a silastic catheter (ID 0.5 mm; OD 1 mm), containing sterile heparinised saline (20 U/ml heparin, 9% saline), was inserted for a distance of 3.5 cm so that the tip lay within the right atrium of the heart. To check that the cannula was in place, a small volume of blood was drawn back prior to the cannula being secured in place with suture thread. The cannula was then

exteriorised at the back of the neck, fixed in placed with a strip of adhesive tape which was sutured to the skin and the cannula was sealed with a plug. The animals were then allowed a minimum of three days to recover.

2.1.2.2 Intraperitoneal injection

The rat was restrained then the needle advanced parallel to the line of the thigh and pushed through to the centre of the posterior quadrant of the abdomen into the peritoneal cavity where the solution was injected.

2.1.2.3 Subcutaneous injection

A fold of the scuff of the neck skin was elevated and a needle was introduced into the raised skin parallel to the body wall. Then the needle was advanced subcutaneously for most of its length and the solution was injected quickly.

2.1.3 Stress

2.1.3.1 Forced swimming

Forced swimming represents a relevant complex physical and emotional stressor for rats (Abel, 1994). With the extension tubing of the venous catheter still attached, rats were forced to swim for 90 s in a red plastic cylinder (40 cm in diameter and 60 cm in height) filled with tap water (19°C) to a depth of about 40 cm. After the swim, the rats were gently dried using towels for 10 s and returned to their home cages.

2.1.3.2 Restraint

Rats were left in a Perspex cylinder (ID: 70 mm) and no free movement for 30 min, then was decapitated. The pituitaries were removed quickly, frozen on dry ice and stored at -70°C until *in situ* hybridisation (Johnstone *et al.* 2000).

2.2 *In vitro* studies

2.2.1 Collection of the hypothalamus and median eminence and CRH assay

On the morning (09:00-10:00) of the experiment, immediately after the rat was decapitated, the median eminence and the hypothalamus were dissected under a binocular microscope. The brain was placed on a glass slide and the median eminence, with the attached pituitary stalk was separated using microscissors. A block containing the hypothalamus was then prepared with a scalpel blade (coronal cuts just anterior to the optic chiasma and caudal to the mammilliary bodies; sagittal cuts along the hypothalamic sulci, and a horizontal cut just ventral to the thalamus). The median eminence and the hypothalamic blocks were snap-frozen on dry ice, and later sonicated in a solution of 0.5 M acetic acid and 0.1 M HCl. The median eminence contents were sonicated in 50 µl solution and the hypothalamus contents were in 250 µl solution. The sonicates were sent by airfreight on dry ice to Prof. Pierluigi Navarra (Catholic University Medical School, Italy) for CRH radioimmunoassay (Dello *et al.* 2000; Navarra *et al.* 1991).

2.2.2 ACTH assay

2.2.2.1 Primary incubation of the rat anterior pituitary cells

Rats were decapitated with a guillotine, the anterior pituitary glands were quickly removed and place in a plastic Petri dish with DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 25 mM HEPES pH7.4, and 0.25% w/v BSA (DMEM-BSA). After the posterior pituitary gland with pars intermedia were removed, the anterior pituitary glands were finely chopped with a straight-edged scalpel blade and then pooled together with other pituitaries within each group. The pituitary pieces were trypsinised by incubating with DMEM-BSA containing 0.25 mg/ml trypsin (TRL) and 10 mg/ml DNase I for 25 min at 37°C, and triturated with a 1 ml Gilson pipette tip attached to the end of a 5 ml polystyrene pipette every 5 min. The tissue suspension was then added to DMEM-BSA containing 0.5 mg/ml Lima Bean Trypsin

inhibitor and 100 kallikrein inhibitor units of aprotinin to inactivate the trypsin. The suspension was then centrifuged at 150 g for 5 min. The supernatant was removed and the pelleted cells were resuspended in DMEM-BSA, gently triturated approx. 35 times with a 1 ml pipette, and filtered through a 100 μm nylon mesh pre-wetted with 4 ml DMEM-BSA. The resulting suspension was then centrifuged at approximate 150 g for 5 min to pellet the cells. The supernatant was carefully removed and the cell pellet was diluted with 1 ml DMEM-BSA. Then the cell suspension was made up to 10 ml with DMEM-BSA and gently rotated for 2 h to allow the cells to equilibrate. Then the suspension was centrifuged at approximate 150 g for 5 min, the cell pellet was re-suspended in 1 ml DMEM-BSA. Cell density was determined with a haemocytometer. Cell viability was assessed by Trypan Blue exclusion and was over 95%. The cell suspension was then diluted to 2 x 10^5 cells/200 μl per tube (Antoni & Dayanithi 1990).

For ACTH secretion studies, the cells were then incubated a 400 μl solution containing different concentrations of ACTH secretagogues (CRH and vasopressin), vehicle (DMEM-BSA), or 0.5 mM 8-CPT-cAMP (a cell permeable cAMP analogue) for 1 h at 37°C in a shaking water bath. In the case of measurement of the effects of the V1a/b receptor antagonist on ACTH secretion by corticotrophs from virgin rats, medium containing [dP(Tyr(Me)^2,Arg-NH_2^{9})AVP was added to tubes containing cells, which were left on ice; 5 min later the secretagogues were then added, and the cells incubated for 1 h at 37°C in a shaking water bath. To measure total ACTH content of cells, 200 μl aliquots (100,000 cells) were frozen directly at –70°C without any incubation or addition of secretagogues.

Each concentration was assayed in 4-6 replicate tubes. The secretion was stopped by placing the tubes on ice for 15 min. Medium containing the secreted ACTH was collected and spun briefly at 150 g to pellet any floating cells and 200 μl supernatant was collected in tubes. The tubes were sealed with parafilm and stored at –70°C until radioimmunoassay.

2.2.2.2 Measurement of ACTH secretion from cell incubation *in vitro*

Duplicate 50 µl aliquots of experimental medium were assayed for immunoreactive ACTH using a double antibody precipitation radioimmunoassay (Dayanithi & Antoni, 1989) after appropriate dilution in RIA buffer (0.05 M sodium phosphate buffer, pH 7.4, 0.1% BSA, 0.1% Triton-X-100, 2.5 mM EDTA and 100 kallikrein inhibitor units of aprotinin per ml) (Antoni & Dayanithi 1990). Lyophilised ^{125}I-ACTH (Phoenix Pharmaceuticals Inc.) was resuspended in distilled water, aliquoted and stored at -20°C. The concentration used gave between 12,000-15,000 cpm/10µl when counted by a gamma counter (LKB-Wallac 1272 Clinigamma with a single detector consisting of a NaI crystal in a 3″ aluminium tube of wall thickness 0.25 mm, Wallac Oy, Finland).

The assay was performed in pre-numbered 0.65 ml polystyrene tubes (LP2 tubes, Luckman Ltd, UK). Fifty microlitres of sheep ACTH antiserum (a kind gift from Prof P.J. Lowry, University of Reading) at a final titre of 1:100,000 in RIA buffer containing 6% polyethylene glycol 6000 (PEG-6000), were mixed with 50 µl of sample. The assay mix was incubated with 10 µl of ^{125}I-ACTH (as above 12,000 to 15,000 cpm in 10 µl sodium phosphate buffer without PEG-6000) for 18 h at 4°C. Subsequently, donkey anti-sheep IgG (Scottish Antibody Production Unit, SAPU, Lanarkshire) and non-immune rabbit serum (SAPU) were added to a final titre of 1:25 and 1:400 respectively and incubated for 3 h at 4°C. Four hundred microlitres of ice-cold 6% PEG-6000 were added and the bound label separated from free by centrifugation at 1,950 g for 25 min at 4°C. The resultant supernatant was decanted, and the radioactivity remaining in the pellet counted on an autogamma counter. Standards were assayed in triplicate. Rat samples were assayed in duplicate.

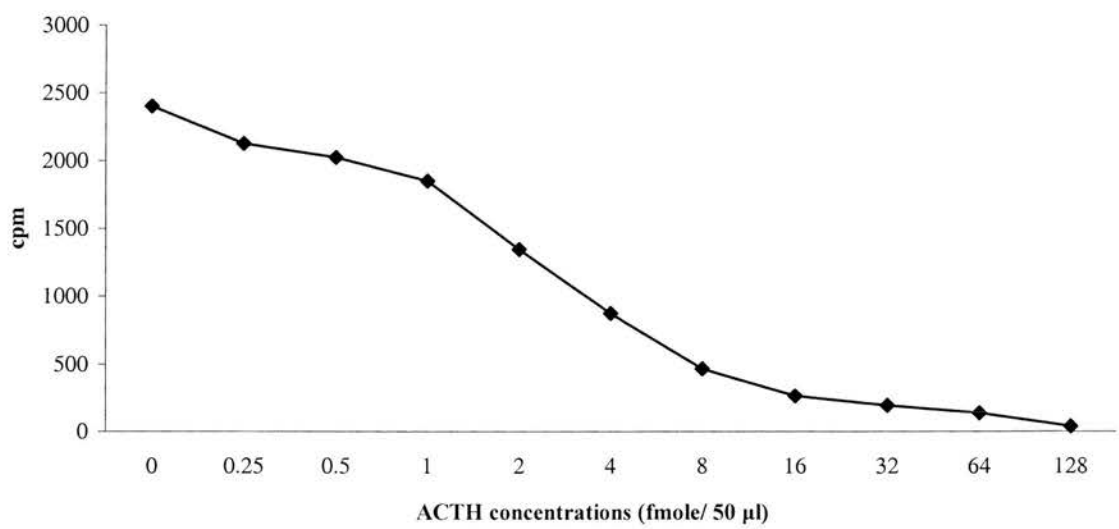
2.2.2.3 Calculation of radioimmunoassay results

To quantify the unknown ACTH concentrations of experimental samples, a standard curve of bound radioactivity against known standard ACTH concentration was compiled in the range 0.125 to 128.0 fmol ACTH/50 µl using human ACTH$^{1-39}$ diluted in RIA buffer as appropriate.

Three other standards were also prepared for construction of the curve and assessing the sensitivity and binding of the assay: total count (TC) contained an aliquot of ^{125}I-ACTH only, to determine total count of radioactivity added; non specific binding (NSB) contained ^{125}I-ACTH, first antibody, second antibody and excess unlabelled ACTH (1280 fmole/50 µl in this study) or by omitting the ACTH antiserum to determine non-specific binding of radioactivity, was typically <10% of the total bound counts; Bo contained aliquots of ^{125}I-ACTH trace, first and second antibody but no unlabelled ACTH, to determine the maximum percentage binding of radioactivity.

A standard curve of bound radioactivity against known standard ACTH concentration was constructed automatically by Wallac Ultroterm 2 software. ACTH contents of inter-assay standards and rat plasma samples were then extrapolated from the standard curve.

Figure 2.1 Standard Curve of bound radioactivity against known standard ACTH concentratsion



The graph above is an example of the standard curve for the calculation of ACTH concentrations.

2.2.2.4 Assay sensitivity

The sensitivity of an assay is the minimum detection limit of an assay. It provides information about the minimum acceptable sample concentration and was calculated using the following equation:

$$\frac{[B_o-(2\times SD)] - NSB}{B_o-NSB}$$

SD is the standard deviation of the ^{125}I-ACTH bound in the three B_O tubes. The value obtained from this equation was then compared to the percentage of B_O radioactivity bound by each standard (0.25-128 fmoles/50µl) calculated using the following equation:

$$\frac{\text{mean standard cpm} - NSB}{B_o - NSB}$$

The sensitivity of the assay was taken at the point where the value obtained using the first equation was greater than those values obtained from the second equation and converted to a concentration in fmoles/50 µl. The assay sensitivity in this study was 0.5 fmoles/50 µl.

2.2.2.5 Measurement of plasma ACTH concentration with an ACTH kit

Plasma ACTH concentrations were measured with an ACTH kit (ICN Biomedicals, Inc., USA). The standard samples and all lyophilised reagents were reconstituted in ddH_2O for 15 min at 4°C before the assays. NSB, contained ^{125}I-ACTH, 0 pg/ml unlabelled ACTH, to determine non-specific binding of radioactivity by omitting the ACTH antibody; B_o, contained aliquots of ^{125}I-ACTH trace, antibody but no unlabelled ACTH, to determine the maximum percentage binding of radioactivity. All Standard concentrations were compiled in the range 10-990 pg/ml and assayed in duplicate. 100 µl of standards or plasma samples, ACTH antibody and human ^{125}I ACTH was added to respective assay tubes. Then the contents of these tubes were shaken thoroughly and

incubated for 16-19 h at 4°C. After incubation, 500 µl precipitant solution was added to all tubes and mixed thoroughly. The supernatant were decanted carefully after being centrifuged at 950 g -1050 g for 15 min. Then the pellet left was counted with a gamma counter.

The method of calculation of radioimmunoassay results is the same as that for measurement of ACTH from incubation (details see 2.2.2.3). The assay sensitivity of this study was 10 pg/ml.

2.2.3 *In situ* hybridisation

Hybridisation is a reaction whereby two single-stranded nucleic acid molecules recognise one another and bind by means of hydrogen bonding of complementary base pairs. *In situ* hybridisation uses a piece of nucleic acid that has been labelled with a radioisotope, or an otherwise detectable molecule, which hybridises to a target nucleic acid residing in a tissue section, permitting determination of the cells expressing that particular gene.

In situ hybridisation histochemistry has become a powerful technique for the detection, localisation and semi-quantification of specific mRNA within morphologically preserved cells or tissue preparations.

2.2.3.1 Choice of probes

Two types of nucleic acid probes in *in situ* hybridisation are commonly used in neuroendocrinology studies with different advantages and disadvantages. Oligonucleotide probes are short (normally 15-50 base pairs long) single-stranded DNA oligonucleotides. These probes are quick and easy to produce without complex molecular knowledge. Their short probe length allows better penetration of the tissue sections, however, it also means that less stringent hybridisation conditions have to be employed leading to a loss of specificity. Riboprobes are single-stranded RNA

molecules produced from a cloned cDNA that is introduced into a specifically designed plasmid transcription system. We chose to use riboprobes for our *in situ* hybridisation studies because there is no problem about reannealing since they are single-stranded, the transcription reaction produces a labeled probe of a fixed length and high specific activity which forms a highly stable hybrid. These probes have a tendency to hybridise to non-specific sites in the tissue sections, however, pre- and post-hybridisation techniques are employed to reduce this. As oligonucleotide probes are easy to use, we also chose in some of the studies when there was no ready riboprobe available.

To be able to determine the specificity of the hybridisation signal of the probe, termed the antisense probe, a heterologous probe, termed the sense probe, which has a similar length, GC content and specific activity as the antisense probe was included in the *in situ* hybridisation studies. The lack of signal with the sense probe verifies that probe binding is a result of its base sequence and not its physical properties.

2.2.3.2 Choice of isotope

We used ^{35}S- to prepare probes for *in situ* hybridisation. ^{3}H, and ^{32}P, each has its own strengths and weaknesses when compared with ^{35}S. ^{3}H has a much lower specific activity and is more expensive despite its slightly higher resolution at the light microscopic level. ^{32}P has a shorter half-life and poorer resolution, especially on film.

2.2.3.3 *In situ* hybridisation with riboprobes

In our experiments, mRNAs were the target, so it is essential that the pretreatments were performed under RNase-free conditions. To prevent RNase contamination solutions were prepared with double-distilled water with diethylpyrocarbonate (DEPC) added and autoclaved. Glassware and slide racks were baked at 250°C for at least 4 h. Gloves were worn throughout the whole procedure.

2.2.3.3a. Slide preparation:

Microscope slides generally need to be subbed in a solution that provides some adherence for the tissue sections, so that they will remain in place during subsequent histological processing. In all the experiments with riboprobe, we used commercially available poly-L-lysine coated slides (BDH, UK). As for the experiments with oligonucleotides, we used the gelatin coated slides.

2.2.3.3b. Tissue collection

After the animals were killed by decapitation, the pituitaries were rapidly removed and immediately frozen on dry ice and stored at –70°C. Coronal sections were cut to 14 μm using a cryostat at –18°C, then mounted onto gelatin or poly-L-Lysine coated slides and stored at –70°C until used for *in situ* hybridisation. Three sections were collected onto each slide.

2.2.3.3c. Tissue preparation

To preserve tissue morphology to the greatest extent possible while maximising access of the probe to the target RNA, sections were fixed with cold freshly made 4% (W/V) paraformadehyde in 0.1 M phosphate buffered saline (PBS: 20 mM NaH_2PO_4, 80 mM Na_2HPO_4, pH 7.4) for 10 min. Paraformadehyde is an alkylating agent which cross-links nucleic acid and protein to form a network within a cell; it provides a superior retention of RNA in sections and maintains the morphology during the long hybridisation and wash procedures. Fixation was carried out at 0-4°C for 10 min to inhibit endogenous ribonucleases. Paraformadehyde fixative was removed by washing 5 min twice in 1 x PBS. Sections were treated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min. The aim of this 'acetylation' protocol is to reduce the non-specific binding of negatively charged nucleic acid to positively charged tissue and microscope slide by acetylating amino groups. The sections were washed in 1x PBS for another 5 min, then dehydrated through a series of ethanols (70%, 80%, 95% prepared with DEPC-treated water, 2 min each); the slides were then air-dried.

2.2.3.3d. Prehybridisation

Prehybridisation is to reduce the background originating from non-specific binding. The tissue equilibrates with components of the hybridisation buffer, and 'sticky' sites are blocked by components such as pyrophosphate and unlabelled RNA or genomic DNA. Prehybridisation was carried out in a saline medium (0.3 M NaCl, 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA pH 7.5, 0.5 x Denhardt's solution, 0.25 mg/ml sheared single stranded salmon sperm DNA, 0.05 mg/ml yeast tRNA and 50% v/v deionised formamide) to reduce non-specific binding sites. Salmon sperm DNA was also been used to cut down the background by binding the chromosomes competing with labelled probe. The slides were placed flat on 3 M Whatman paper soaked in box buffer (50% deionised formamide, 20% 20 x SSC (3 M NaCl and 0.3 M sodium citrate pH 7.0) and 30% DEPC-treated water) in a humidified chamber, and then incubated with 200 µl 1 x prehybridisation solution for 2 h at 50°C.

2.2.3.3e. Hybridisation

After prehybridisition, the slides were dried around the tissue with lens paper. To each slide was carefully added 200 µl hybridisation solution. Hybridisation solution comprised 50% (v/v) of deionised formamide, radiolabeled probe (10 x 10^6 cpm/ml) mixed with 2 x hybridisation solution (0.6 M NaCl, 10 mM Tris-Hcl pH 7.5, 1 mM EDTA pH 7.5, 1 x Denhardt's solution, 0.1 mg/ml denatured salmon sperm DNA, 0.1 mg/ml yeast tRNA and 10% dextran sulphate), mixed at 70°C for 10 min. Briefly cooled on ice, 1 M Dithiothreitol (10 µl/ml of probe) was added to this mixture. Dextran sulphate may be useful to accelerate the rate of probe association and hence effectively increasing probe concentration. After adding 200 µl hybridisation solution, all slides were placed in a humidified hybridisation box, sealed and incubated overnight (around 16 h) at 50°C.

2.2.3.3f. Posthybridisation wash

After hybridisation incubation, washing of the hybridised sections was carried out to remove probe that had bound to sequences related to, but distinct from, the intended target (for example, for mRNA conserved gene families) or non-specifically to other cellular components. The critical requirement is use of stringency conditions to remove only the non-specifically bound probe and treatment of the sections with RNAse A is also important to reduce background. The slides were washed in a total of 3 changes of 2 x SSC (5 min each) at room temperature. Immediately after this, 200 µl of RNase A solution (30 µg/ml in RNase buffer containing 10 mM Tris-HCl pH 7.5, 1mM EDTA pH 7.5 and 0.5 M NaCl in ddH_2O) was applied to each slide. The slides were incubated at 37°C for 1 h, afterwards, the slides were progressively washed with more stringency, beginning with a 30 min wash in 2 x SSC at room temperature. Subsequently slides were washed in 0.1 x SSC at 60°C for 90 min followed by another wash in 0.1 x SSC starting at 60°C and allowed to cool to room temperature. The slides were then dehydrated in a series of 50%, 70% and 90% ethanol in 0.3 M ammonium acetate for 2 min each and allowed to air dry.

2.2.3.3g. Visualisation of signal

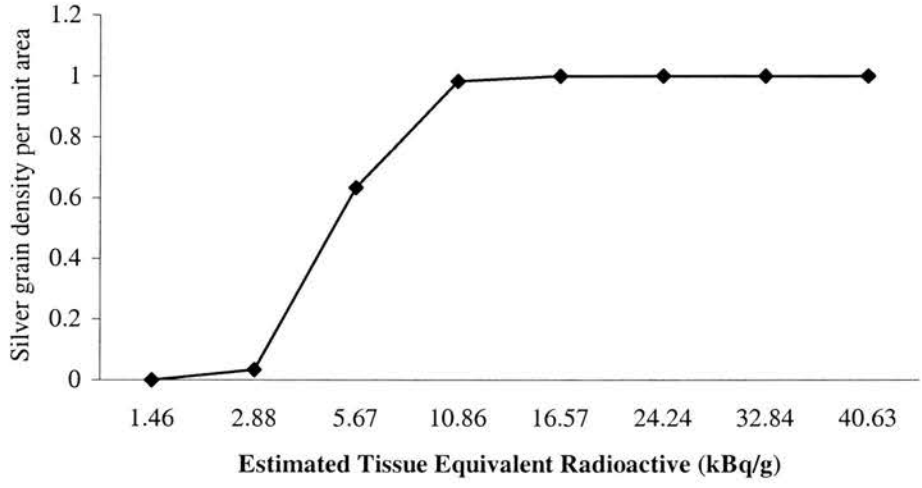
Following post-hybridisation washing, the slides were apposed against Hyperfilm-β max for an appropriate time determined for each probe. The films were developed with D-19 for 5 min, rinsed briefly in ddH_2O, fixed in a 1:4 dilution of Ilford Hypam rapid fixer for another 5 min and washed in ddH_2O for 30 min. All development steps were carried out at 15°C in a dark room.

2.2.3.3h. Quantification of autoradiographs

Analysis of autoradiographic film images was performed with a microscope (objective x 5) linked to a computer based image analysis system. Film grain density was measured (5x obj. in a 0.53 x 0.53 mm and 0.15 x 0.15 mm frame for the sections from rats and mice respectively), placed over 2 regions per section. For each animal,

measurements were made on six replicate sections. Background measurements were made on areas adjacent to the pituitary and subtracted. The results are expressed as silver grain density (ie. grain area per mm^2).

Figure 2.2 Radioactive Curve



Standards curve of the film grain density was obtained by exposing the ^{14}C polymer strip (Amersham Pharmacia Biotech, UK) standard slides along with the samples for the same time. The graph above is one example of estimated tissue equivalent radioactivity and film grain density after film was exposed to the ^{14}C standard slide for 14 days. In all *in situ* hybridisation slides we measured, grain density was in the dynamic slope part of the standard curve, where the density changes quickly with the change of quantity of labelled radioactive.

2.2.3.3.1 Preparation of competent E.coli: for riboprobe preparation

Bacteria treated with ice-cold solutions of $CaCl_2$ and briefly heated were used to transform bacteria with plasmid DNA (Cohen *et al.*, 1972). This treatment induces a transient state of "competence" in the recipient bacteria, during which they are able to take up DNAs derived from a variety of sources.

DH 5α (parental E Coli strain) was streaked from a stock onto a LB agar plate (Lennox L Agar 35 g/L in ddH_2O, autoclaved and cooled to form gel in the petri plates), and incubated for 16 h at 37°C. Several well-isolated colonies were transferred into 250 ml autoclaved 2 x TY (bacto-tryptone 1.6%, bacto-yeast extract 1% and NaCl 0.5%, pH 7.0 in ddH_2O). The cells were grown in a shaking incubator at 37°C for 2-2.5 h until they were in the mid-log phase detected as cloudiness and cloudy on swirling. Then this solution was poured into four 50 ml falcon tubes, centrifuged at 3000 rpm for 10 min at 4°C, supernatant removed and pellet re-suspended in 250 ml ice cold 50 mM $CaCl_2$. The solution was cooled on ice for 15 min, centrifuged as before and re-suspended in 20 ml ice cold freezing mix (100 mM KCl, 50 mM $CaCl_2$, 10% glycerol (w/v), 10 mM KOAc, autoclaved, pH 7.5). 200 µl aliquots were taken into sterile screw-cap Eppendorfs placed on dry ice and stored at –70°C until use.

2.2.3.3.2 Transformation of competent E.coli

About 0.5 µg plasmid DNA was added into 200 µl competent cells (DH-5α E.coli) in an incubation tube and mixed by swirling the tube gently several times and placed on ice for 30 min (during this stage the cells take up the DNA). The tube was held in a water bath (42°C) for 75 seconds (heat shock) and rapidly transferred onto ice to cool down the contents to room temperature. 0.4 ml 2 x TY was added to the tube and incubated in a shaking incubator for 30 min at 37°C. After incubation, 200 µl cells were transferred onto an Ampicilllin plate (Lennox L Agar 35 g/L in ddH_2O, and 50 mg/ml Ampicillin, pH 7.0 in ddH_2O, autoclaved and cooled to form gel state). This plate was incubated upside-down at 37°C for 12-16 h.

2.2.3.3.3 Extraction and preparation of plasmid DNA

The extraction of plasmid DNA was achieved by using a commercially available kit (QIAgen, Germany). Briefly, after incubation, a single colony was picked from the plate above and incubated first in 1.5 ml 2 x TY solution for 4-5 h, then transferred to and grown in 50 ml autoclaved 2 x TY solution in a shaking incubator (300 rpm) overnight. On the following morning the bacteria cell solution was centrifuged at 6000

g for 15 min at 4°C. The supernatant was carefully removed and the bacterial pellet re-suspended in 4 ml of buffer P1 (50 mM Tris.Cl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and mixed well by pipetting. Then 4 ml buffer P2 (200 mM NaOH, 1% SDS) was added, mixed gently by inverting 4-6 times and this mixture was incubated at room temperature for 5 min. Then 4 ml pre-chilled (4°C) buffer P3 (3 M potassium acetate, pH 5.5) was added, mixed immediately and gently, and incubated on ice for 15 min. After this incubation, the solution was centrifuged at 20,000 g for 30 min at 4°C and the supernatant was re-centrifuged at 20,000 g for 15 min at 4°C. The supernatant was applied to an equilibrated QIAgen-tip 500 (by a solution containing 750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol), washed twice with 10 ml buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). Plasmid DNA was then eluted with 5 ml of buffer QF (1.25 M NaCl, 50 mM Tris.Cl, pH 8.5, 15% isopropanol), precipitated by adding 3.5 ml of isopropanol and centrifuged at 15,000 g for 30 min at 4°C. The DNA pellet was washed with 2 ml 70% ethanol and centrifuged again at 15,000 g for 10 min, then the supernatant was carefully removed. The pellet was air-dried, dissolved in an appropriate volume of ddH_2O and stored at -20°C. The concentration of plasmid was determined by measuring the absorbance at 260 nm wavelength (1 OD260 = 50 µg/ml DNA).

2.2.3.3.4 Plasmid linearisation

The double-stranded DNAs were digested with a restriction enzyme to generate the linear DNA. The resulting single-stranded DNAs were used as a template. Plasmid DNA templates were completely cleaved, since trace amounts of supercoiled plasmid DNA result in the generation of extremely long transcripts that include plasmid sequences. These long transcripts incorporate radiolabel and increase background hybridisation.

Rat glucocorticoid receptor (rGR)

The pGEM3 plasmid containing a 620 bp fragment of rat glucocorticoid receptor (rGR) cDNA was linearised by incubation with either Ava I or EcoR I to generate the

antisense or sense DNA fragments, respectively, for 2 h at 37°C. The plasmid was a kind gift from Prof. JR.Seckl, Edinburgh, UK (Miesfeld *et al.*, 1986).

Rat corticotropin-releasing hormone receptor 1 (rCRHR1)

The pGEM4Z plasmid containing a 606 bp fragment of rat corticotropin-releasing hormone receptor 1 (rCRHR1) cDNA was linearised by incubation with either Hind III or EcoR I to generate the antisense or sense DNA fragments, respectively, for 2 h at 37°C. The plasmid was a kind gift from Dr.Stephen J.Lolait, Bristol, UK (Perrin *et al.*, 1993).

Rat vasopressin receptor 1b (rV1b)

The Bluescript II KS (+/-) plasmid containing a 464 bp fragment of rat vasopressin receptor 1b (rV1b) cDNA was linearised by incubation with either Hind III or EcoR I to generate the antisense or sense DNA fragments, respectively, for 2 h at 37°C. The plasmid was a kind gift from Dr.Stephen J. Lolait, Bristol, UK (Lolait *et al.*, 1995).

Human corticotropin-releasing hormone-binding protein (CRHBP)

The Bluescript II KS (+/-) plasmid containing a 969 bp fragment of human Corticotropin-releasing hormone-binding protein (hCRH-BP) cDNA was linearised by incubation with either Xho I or Sac II to generate the antisense or sense DNA fragments, respectively, for 2 h at 37°C. The plasmid was a kind gift from Prof. P. Lowry, Reading, UK (Potter *et al.*, 1991).

2.2.3.3.5 Phenol/chloroform extraction of plasmid

To remove proteins (enzymes), the templates were purified by extracting with phenol:chloroform. To the template in a 1.5 ml Eppendorf tube, an volume equal to the plasmid solution of phenol:chloroform:alcohol (24:25:1) was added, mixed vigorously by inversion. The mixture was centrifuged at 12000 g for 5 min, the aqueous (upper, containing DNA) phase was carefully removed and transferred to another 1.5 ml Eppendorf. One volume phenol:chloroform (24:25) was added to this DNA solution,

mixed vigorously by inversion, and centrifuged at 12000 g for 5 min. The upper aqueous phase was removed carefully to a fresh Eppendorf tube, 0.1 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of absolute ethanol were added, and mixed by inversion. This mixture was frozen on dry ice for 10 min. Then it was thawed and centrifuged at 12000 g for 15 min and the supernatant was removed. The pellet was air dried and re-suspended in appropriate volume of DEPC-treated H_2O; 1 µl of the DNA was electrophoresed on 1% agarose gel to check the purity of the linearised plasmid. The remaining template was stored at –20°C.

2.2.3.3.6 *In vitro* transcription of ^{35}S-UTP labelled cRNA probe

Sense and anti-sense hybridisation were used as a control procedure for *in situ* hybridisations with riboprobes. Riboprobes were synthesised by transcription using cDNA as a template. Antisense riboprobes were from cDNA inserts subcloned in the transcription vector in the 3' to 5' direction relative to an RNA polymerase initiation site. The anti-sense transcribed in the presence of a labelled nucleotide probe is complementary to the cellular mRNA and binds to it during the hybridisation incubation. Insertion of the cDNA in the opposite orientation relative to the RNA polymerase initiation site transcribes the control sense riboprobe, a sequence identical to the mRNA in the tissues which does not hybridise to this mRNA. As the sense and anti-sense probes contain the same relative proportion of all of the nucleotides (A/Us or G/Cs) in the same sequence, sense probe is an appropriate control (Cox *et al.*, 1984). 1 µg of each of the linearised templates was incubated with 1.5 µl of mixed cold nucleotides (10 mM of ATP, CTP and GTP), 0.5 µl of 200 mM freshly-made DTT, 0.5 µl of RNase inhibitor, 4 µl of ^{35}S-UTP (40 mCi/1 ml), 1 µl of the specific RNA polymerases in the presence of 5 x transcription buffer in a volume of ~10 µl. The reaction was carried out in an RNase-free Eppendorf tube. The incubation was carried out at 37°C for T3 and T7, and 40°C for SP6 polymerases for approximately 1 h. After the incubation, 1 µl of RNase-free DNase was added to the reaction mixture and incubated for a further 15 min at 37°C. The reaction was stopped by placing the tube on ice. The newly synthesised probe was purified through a Nick column (Sephadex G

50). The column was first washed with 3 ml of TE, pH 8.0 in order to equilibrate the gel bed. The reaction mix was applied to the column followed by 400 µl of TE buffer was applied to the column and the eluant collected, as this contained the labelled probe.

2.2.3.3.7 Agarose gel electrophrosis

1% agarose was added to100 ml 1x TBE buffer (0.09 M Tris-borate, 0.002 M EDTA, pH 8.0) in a baked flask and heated to dissolve the agarose. The solution was cooled to 55°C, and 3 µl of 10 mg/ml ethidium bromide was added. Enough 1x TBE buffer was added to cover the gel to a depth of about 1 mm. DNA samples mixed with gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in water) were loaded into the slots of the submerged gel. DNA migrated toward the anode under an applied voltage of 100 mV. Electrophoresis was stopped when the bromophenol blue and xylene cyanol FF migrated the appropriate distance through the gel. The DNA bands in the gel were examined by ultraviolet light.

2.2.3.3.8 Polyacrylamide gel electrophoresis

To check the purity of radiolabelled riboprobes, we ran polyacrylamide elecrophoresis and only those labelled probes showing clear bands on the film were used in *in situ* hybridisation.

We prepared 10 ml 4% polyacrylamide gel with a SequeGel kit by mixing 1.6 ml SequaGel Concentrated solution (23.75% w/v acrylamide, 1.25% w/v methylene bisacrylamide and 8.3 M urea), 7.4 ml SequaGel Diluent solution (8.3 M urea), 1 ml SequaGel Buffer (8.3 M urea in 1.0 M tris-Borate-20 mM EDTA buffer pH 8.3), 80 µl 10% (w/v) ammonium persulfate and 4 µl Temed. Gels were run for 2 h at 120 V in 1 x TBE buffer (0.09 M Tris-borate, 0.002 M EDTA, pH 8.0) and autoradiographed.

2.2.3.4 *In situ* hybridisation with oligonucleotides

The method for *in situ* hybridisation with oligonucleotides is slightly different from that for riboprobes. POMC antisense oligonucleotides were 48 base pairs complementary to

part of the mRNA sequences coding for POMC peptides (Nakanishi *et al.* 1981). 48 base pairs of BK antisense oligonucleotides that were randomly complementary to the mRNA sequences coding for non-STREX of BK (Shipston *et al.* 1999) were selected. 48 base pairs of STREX antisense oligonucleotides that were randomly complementary to the mRNA sequences coding for STREX splice of BK (Shipston *et al.* 1999) were selected. As the technique of *in situ* hybridisation with the POMC oligonucleotide probe is well established in our lab, we did not use a sense probe as a control in this study. In BK and STREX mRNA measurements, we employed sense oligonucleotide probe as control.

2.2.3.4.1 Preparation of coated slides and tissue collection

Slides were treated in chromic acid (200 g potassium dichromate, 2000 ml ddH_2O, to which 20 ml concentrated sulphuric acid slowly added) overnight, then rinsed completely with cold running water for a few hours. Slides were mounted into racks, rinsed in three changes of ddH_2O then once in DEPC-treated ddH_2O (1 ml diethyl pyrocarbonate, 1000 ml ddH_2O, autoclaved), dipped in 80% alcohol (in DEPC treated water). The slides were dried in a 60°C oven and cooled to room temperature, then dipped in chrome–alum/gelatin subbing solution (2.25 g gelatin and 0.23 g chromic potassium sulphate in 800 ml distilled water) for 2 min, and placed on clean paper towelling to drain and air-dried. The next day, each rack of slides was dipped briefly in the subbing solution again. Dried slides were stored in dry, clean slides boxes until required. The method of tissue collection for oligonucleotide probe was the same as that for riboprobe (see 2.2.3.3c)

2.2.3.4.2 3'-end labelling of oligonucleotide probe

3' terminal labelling utilises the ability of terminal deoxynucleotidyl transferase (TdT) to add on deoxyribonucleotides to the 3' ends of oligonucleotides. This allows the addition of labelled nucleotides. In this study, we use a terminal transferase kit to label the oligonucleotide probes. 27 µl ddH_2O, 10 µl 5 x TdT buffer, 5 µl $CoCl_2$, 5 µl ^{35}S dATP and 2 µl oligonucleotides were mixed in a 0.5 ml Eppendorf tube, and 1 µl TdT

enzyme added. The mixture was ensured by pippeting and was incubated in a 37°C water bath for 1 h.

2.2.3.4.3 Purification of radiolabelled oligonucleotides

The radiolabelled oligonucleotides were purified with a QIAquick nucleotide removal kit. The Eppendorf tube containing radiolabelled oligonucleotide probe was removed from the water bath and 500 µl PN buffer added. The contents of the eppendorf were transferred transfer to a spin column placed inside a 2 ml centrifuge tube. The solution was centrifuged at 6000 rpm for 1 min. The inner column was remove and put into a fresh centrifuge tube, 500 µl PE buffer was added and centrifuged at 6000 rpm for 1 min. Another 500 µl PE buffer was added and centrifuged at 13000 rpm for 1min. The spin column was placed in an autoclaved eppendorf tube and 50 µl ddH_2O was carefully added to cover the resin filter in the base of the column. The labelled probe was eluted by centrifugation at 13000 rpm for 1 min. The labelled probe was counted by counting a 2 µl sample in 3.5 ml scintillation fluid with a β- scintillation counter and stored at -20°C.

2.2.3.4.4 Prehybridisation

Cryostat cut pituitary sections were removed from the –70°C freezer in their boxes (keeping the lids on to prevent condensation) and dried at room temperature for 1-2 h. The dry slides were placed in racks and passed through the following series: 5 min in 4% formaldehyde in 0.1 M PBS (pH 7.2), 5 min in 0.1 M PBS twice, 10 min in triehanolamine/acetic anhydride (0.75 ml acetic anhydride, 4.47 ml triethanolamine, 1.26 ml 10 M HCl in 300 ml autoclaved ddH_2O), 2 min in ddH_2O, 2 min in 70% ethanol, 2 min in 95% ethanol, 1 min in 100% ethanol, 5 min in chloroform to delipidate tissue, 2 min in 100% ethanol, 1 min 95% ethanol, then air-dried thoroughly.

To each slide was carefully added 45 µl hybridisation solution followed by incubation overnight in a sealed humidified hybridisation box at 37°C. Hybridisation solution comprised 50% (v/v) of deionised formamide, radiolabeled probe (0.1×10^6 cpm per

pituitary section) mixed with 2 x hybridisation solution (1.2 M NaCl, 10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 7.5, 1 x Denhardt's solution, and 2.5% dextran sulphate, DTT, 0.1 mg/ml denatured salmon sperm DNA, 0.1 mg/ml yeast tRNA, 0.1 mg/ml yeast tRNA, 0.1 mg/ml yeast total RNA, 0.1 mg/ml poly (A), and 50% v/v formamide).

2.2.3.4.5 Posthybridisation and visualisation

After hybridisation incubation, washing of the hybridised sections was carried out. The slides were washed in four changes of 1 x SSC at 45°C (15 min each). Subsequently slides were washed in 1 x SSC at room temperature for 30 min twice, followed by rinsing in ddH_2O and air-dried.

Visualisation of signal and Quantification of autoradiographs for oligonucleotide probes are the same as for riboprobes.

Chapter 3

In situ hybridisation studies of selected mRNA species related to corticotroph function in the anterior pituitary in pregnancy

3.1 Introduction

CRH and vasopressin are the two major ACTH secretagogues from the hypothalamus and their actions are mediated by CRHR1 and V1b receptors on the corticotrophs respectively. Vasopressin alone is a less effective stimulator of ACTH secretion in rodents, but is the most potent among the co-secretagogues that acts synergistically with CRH to induce ACTH secretion from the anterior pituitary (Gillies *et al.* 1982). Previous studies indicate reduced CRH receptor binding to corticotrophs in pregnancy (Neumann *et al.* 1998). Decreased vasopressin binding to the anterior pituitary has been reported at the end of pregnancy (Toufexis *et al.* 1999), but CRHR1 and V1b receptor mRNA expression has not been measured.

The inhibitory effects of corticosterone on HPA axis function are believed to be transduced primarily by intracellular steroid receptor proteins that function as hormone-activated transcription factors, GR and MR. In the anterior pituitary, GR is the main receptor to mediate the negative feedback effects of corticosterone (Herman *et al.* 1989; Ozawa *et al.* 1999). There is already an indication, from measurement of increased 11β-HSD activity in the anterior pituitary in pregnancy (Johnstone *et al.* 2000), of enhanced corticosterone negative feedback. Increased GR expression could contribute to increased feedback sensitivity. Measurement of changes in anterior pituitary POMC mRNA expression after removal of corticosterone would be valuable to understand negative feedback exerted by glucocorticoids during pregnancy.

The POMC gene encodes a common precursor protein for a family of peptides including ACTH (Lowry *et al.* 1980; Chretien & Seidah 1981; King & Baertschi 1990). In corticotrophs, POMC is cleaved into ACTH, β-lipotropin (β-LPH) and small amounts of β-endorphin (Lazarus *et al.* 1976; Lowry *et al.* 1980; Chretien & Seidah 1981; Lundblad & Roberts 1988; King & Baertschi 1990). Reduced POMC gene expression in corticotrophs in pregnancy might underlie the reduced ACTH secretory response.

In corticotrophs, CRHBP is mainly associated with secondary lysosomes, multivesicular bodies, and endosome-like vesicular structures. This suggests that CRHBP is in a position to participate in the signal transduction of the CRH-CRH receptor complex. CRHBP facilitates the dissociation of the complex, with consequent freeing of the receptor for recycling to the cell surface, and/or acting as chaperone to direct internalised CRH to the lysosomal system of digestion (Peto *et al.* 1999). CRHBP is not in secretory granules in the pituitary cells, suggesting that it is not co-secreted with ACTH (Peto *et al.* 1999). Altered CRHBP expression might lead to altered CRH action on corticotrophs in pregnancy.

The release of ACTH induced by CRH from the corticotrophs is largely dependent on cAMP-mediated stimulation of Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels (Luini *et al.* 1985; Guerineau *et al.* 1991). CRH-induced membrane depolarisation is due, in part, to inhibition of large-conductance Ca^{2+}-activated K^{+}-channel (BK-channel) by PKA. The secretion of ACTH stimulated by vasopressin through the IP pathway requires extracellular Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels (Nishizuka 1984; Won *et al.* 1990). This increased intracellular Ca^{2+} then triggers the ACTH secretion from the corticotrophs. A splice variant of the BK channel α-subunit, stress axis regulated exon (STREX), was identified and subsequently cloned in AtT20 cells (Shipston *et al.* 1999). The α-subunit containing this splice variant is inhibited by cAMP-mediated protein phosphorylation. In AtT20 cells, about 90% of endogenous BK channels are comprised of the high calcium sensitive STREX splice variant. However, the proportions of the BK channels containing the STREX splice in rat corticotrophs, and the changes of this proportion in the pregnancy state, are not known.

3.2 Aims

During pregnancy, the ACTH response(s) to stress are attenuated, which may be partly due to changes in corticotroph sensitivity, including sensitivity to decreased stimulatory effects and/or increased inhibitory effects. So the knowledge of expression of the factors that mediate stimulatory (CRHR1, V1b receptor), or inhibitory (GR, CRHBP, BK channels and STREX) responses in the corticotrophs is valuable to understand the mechanism of ACTH secretion regulation during pregnancy. Measurement of the expression of POMC gene which encodes the precursor of ACTH would provide some evidence of changes in ACTH synthesis, and thus secretion.

In this study, to seek changes in expression of mRNA species that may underlie altered responsiveness of corticotrophs to secretagogues in pregnancy, we measured the mRNAs of these proteins by *in situ* hybridisation.

3.3 Materials and methods

The animals were housed in the animal facility (MFAA) in the University of Edinburgh Medical School. For details see Chapter 2.1.1. For CRHR1, V1b receptor, GR, POMC, BK and STREX mRNA measurement, individually housed virgin, day 10 and day 21 pregnant rats, or virgin and day 18 pregnant mice were transferred separately to the post mortem room where they were decapitated between 09.30 h and 10.30 h to minimise the acute effects of stress. The pituitaries were rapidly removed, immediately frozen on dry ice and stored at –70°C. CRHBP attenuates ACTH secretion stimulated by CRH (Behan DP 1989; Linton *et al.* 1990), so it is valuable to investigate whether CRHBP expression is changed in pregnant rats at basal level compared with in virgin rats. The changes of CRHBP mRNA are very rapid in the anterior pituitary (significantly increases with 30 min) (McClennen *et al.* 1998), so it permits us to measure this gene expression after stress in the morning. Virgin and pregnant (day 21) rats were restrained in a cylinder (ID: 70mm) for 30 min between 08.00h and 10.30 h. One hour after restraint stress, all rats were killed and pituitaries

were removed, frozen on dry ice and stored at -70°C. Control rats were left in their cages and killed at the same time when the stressed rats were killed.

Enhanced corticosterone negative feedback may exist in pregnant rats (Johnstone *et al.* 2000). To test whether attenuated ACTH response to stress is due to a greater inhibition of POMC mRNA expression by elevated plasma corticosterone concentration in late pregnancy, we removed endogenous corticosterone by pharmacological adrenalectomy (phADX) (Plotsky & Sawchenko 1987). The pituitaries were taken from virgin and pregnant rats prepared and treated by Paula Brunton. To block glucocorticoid synthesis the rats were administered either 100 mg/kg metyrapone or saline vehicle subcutaneously at 8 h intervals over a 48 hour period (beginning at 08.00 h on day 19 of pregnancy). The final metyrapone injection (at 08.00 h on day 21 pregnancy) was followed 75 min later by a subcutaneous injection of 200 mg/kg aminoglutethimide/dimethyl-sulphoxide) (Johnstone *et al.* 2000). Controls received vehicle injection. One hour after the last injection, all rats were killed and pituitaries were removed and frozen on dry ice for *in situ* hybridisation.

Coronal sections were cut at 14 μm using a cryostat at –18°C, then mounted onto gelatin (for oligonucleotide probes: POMC, BK and STREX) or poly-L-Lysine (for riboprobes: CRHR1, V1b receptor, GR, CRHBP) coated slides and stored at –70°C until *in situ* hybridisation. Details of *in situ* hybridisation with oligonucleotide probes or riboprobes, and film autoradiograph silver grain counting are in Chapter 2.2.3.

Statistics

Data were expressed as silver grain density mean ±SEM. Data from CRHR1, V1b receptor, GR, BK and STREX mRNAs were analysed with t-tests. Data from POMC and CRHBP mRNAs were analysed with one-way ANOVA followed by post-hoc Student-Newman-Keuls tests.

3.4 Results

The signal in the sections incubated with the labelled sense probe was always much less than that with the respective antisense riboprobe or oligonucleotide probe.

CRHR1 mRNA

CRHR1 mRNA expression (Figures 3.1a and 3.1b) in pituitaries from virgin and day 21 pregnant rats was measured by *in situ* hybridisation with a radiolabelled riboprobe. There was signal in the anterior pituitary, and strong signal in the intermediate lobe, but no signal in the posterior pituitary. The silver grain densities in virgin and day 21 pregnant rat anterior pituitaries were not different (t-test, p=0.92; virgin, n=9; pregnant, n=7).

V1b receptor mRNA

With a radiolabelled probe corresponding to the 5' end UTR sequence of the V1b receptor gene, V1b receptor mRNA expression reflected by silver grain density was detected to be in the anterior pituitary. By semi-quantitative measurement, V1b receptor mRNA expression was decreased by 19% in the anterior pituitary from day 21 pregnant rats (Figures 3.2a and 3.2b, t-test, *p<0.05 *vs* virgin, t-test; virgin, n=9; pregnant, n=7).

GR mRNA

GR mRNA was found abundantly in the anterior and intermediate pituitary, and expression in the anterior pituitary in pregnant rats was increased by 26% compared with that of the virgins (Figures 3.3a and 3.3b, t-test, *p<0.05 *vs* virgin, t-test; virgin, n=6; pregnant, n=5).

Figure 3.1a Anterior pituitary CRHR1 mRNA expression in rats

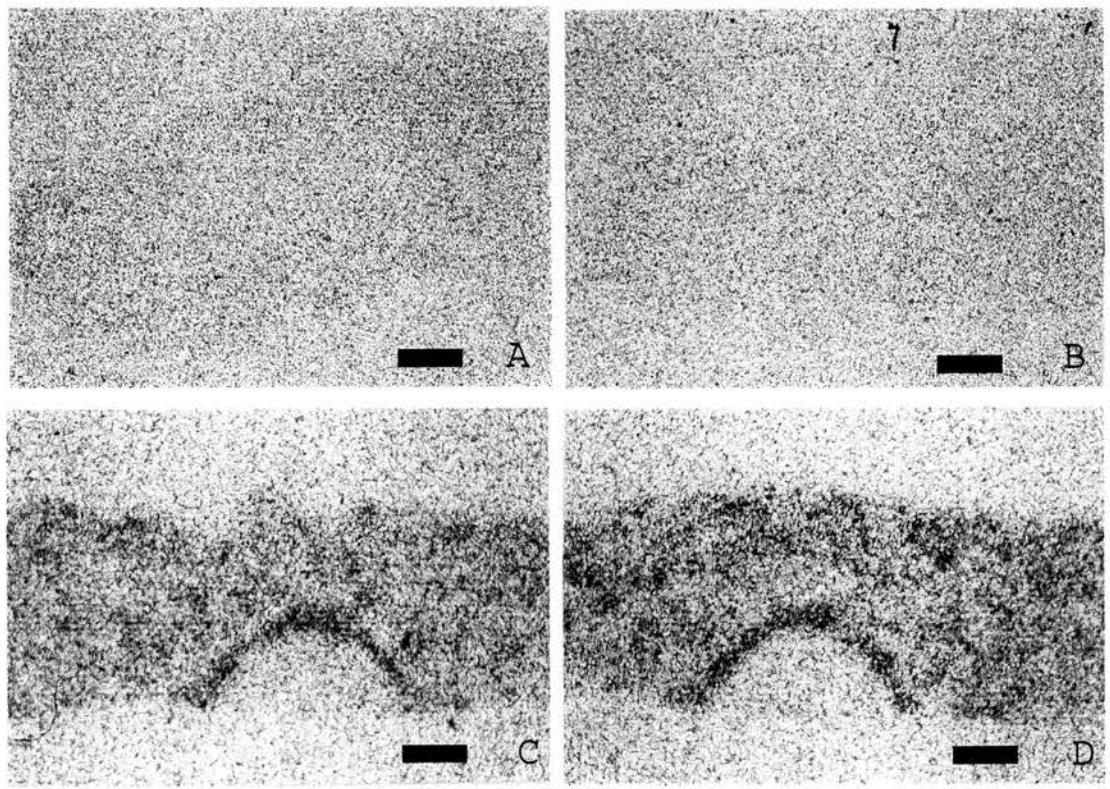


Figure 3.1a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense riboprobes for CRHR1 mRNA. A and B were hybridised with labelled sense riboprobe from virgin and day 21 pregnant rats respectively; C and D were hybridised with labelled antisense riboprobe from virgin and pregnant rats respectively. Scale bar = 0.5 mm.

Figure 3.1b Anterior pituitary CRHR1 mRNA expression in rats

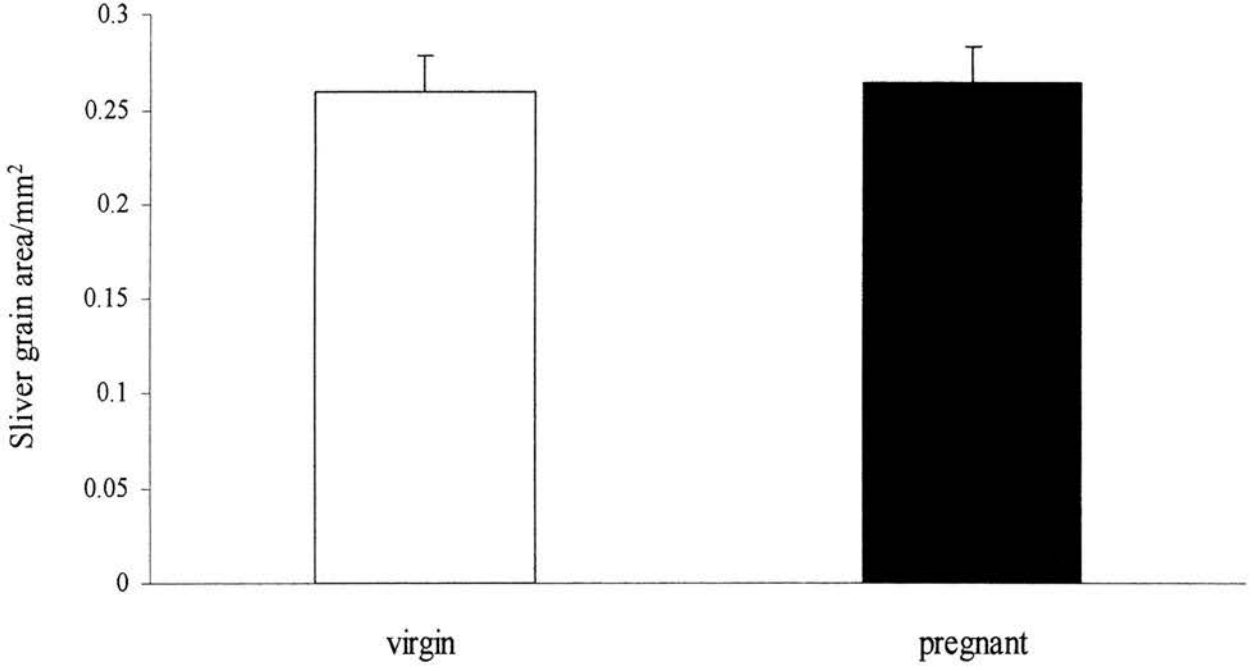


Figure 3.1b. CRHR1 mRNA expression in the anterior pituitary collected from day 21 pregnant and virgin rats. Film autoradiographs of pituitary sections probed with a ^{35}S riboprobe for CRHR1 mRNA were analysed by a computer-based image analysis system to measure area of silver grains, and the data expressed as mean±SEM silver grain density over a unit area. There was no difference between virgin and pregnant rats (n=9, 7 for virgin and pregnant respectively, t-test: *p>0.05).

Figure 3.2a Anterior pituitary V1b receptor mRNA expression in rats

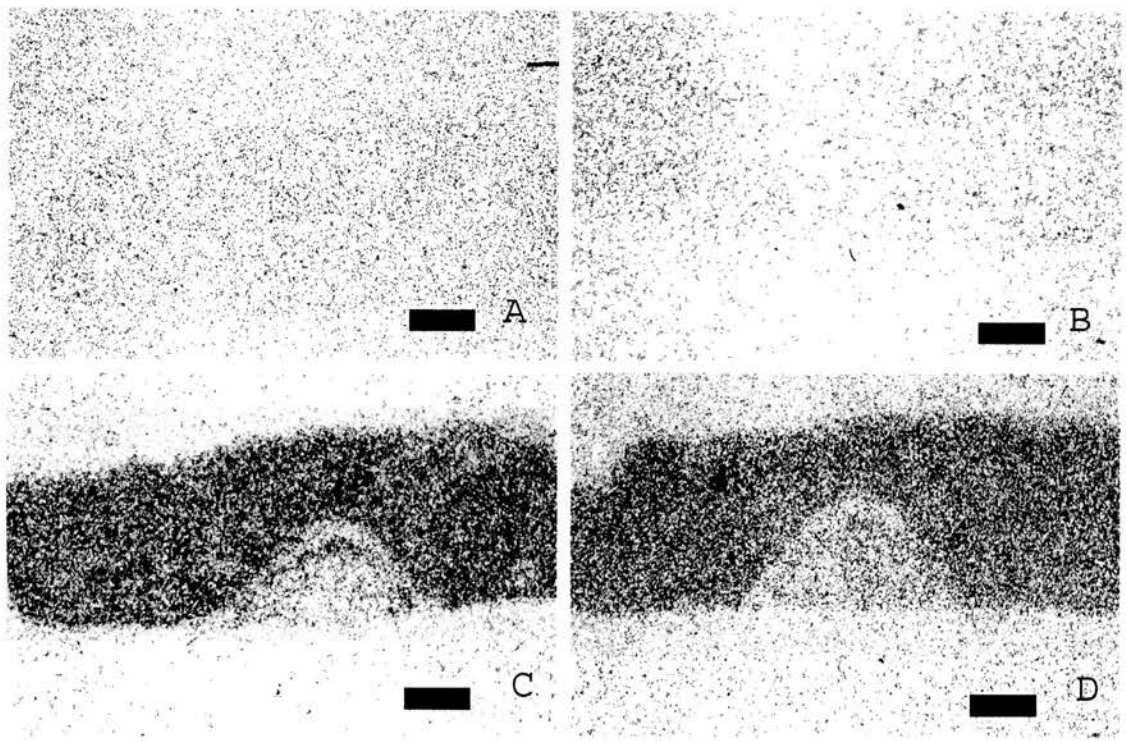


Figure 3.2a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense riboprobes for V1b receptor mRNA. A and B were hybridised with labelled sense riboprobe from virgin and day 21 pregnant rats respectively; C and D were hybridised with labelled antisense riboprobe from virgin and pregnant rats respectively. Scale bar = 0.5 mm.

Figure 3.2b Anterior pituitary V1b receptor mRNA expression in rats

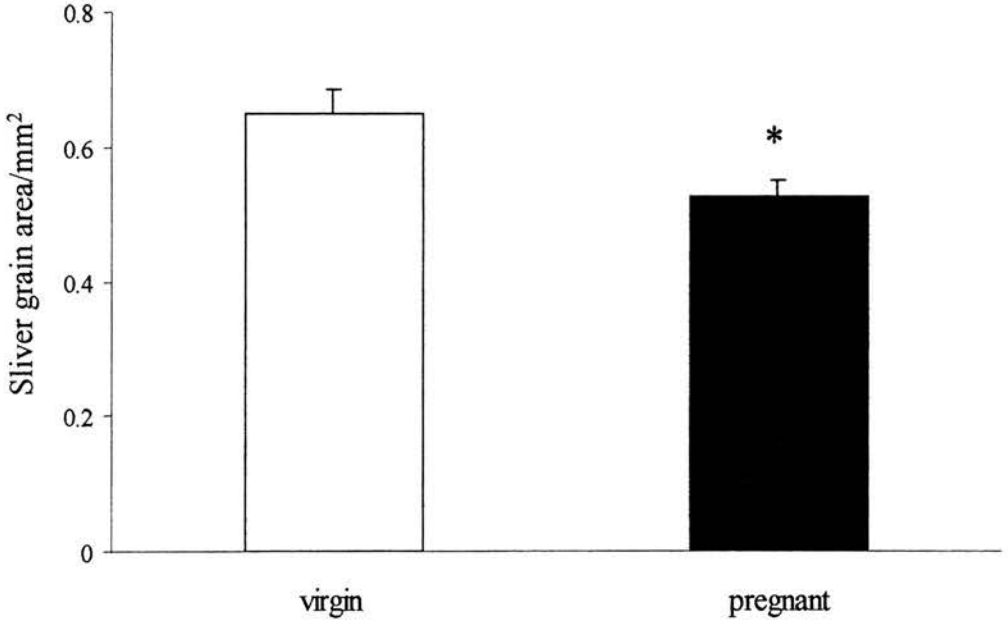


Figure 3.2b. V1b receptor mRNA expression in the anterior pituitary collected from virgin and day 21 pregnant rats. Film autoradiographs of pituitary sections probed with a ^{35}S riboprobe for V1b receptor mRNA were analysed by a computer-based image analysis system to measure area of silver grains, and the data expressed as mean±SEM silver grain density over a unit area. Anterior pituitary V1b receptor mRNA expression in pregnant rats was reduced (n=9, 7 for virgin and pregnant respectively. t-test: *$p<0.05$)

Figure 3.3a Anterior pituitary GR mRNA expression in rats

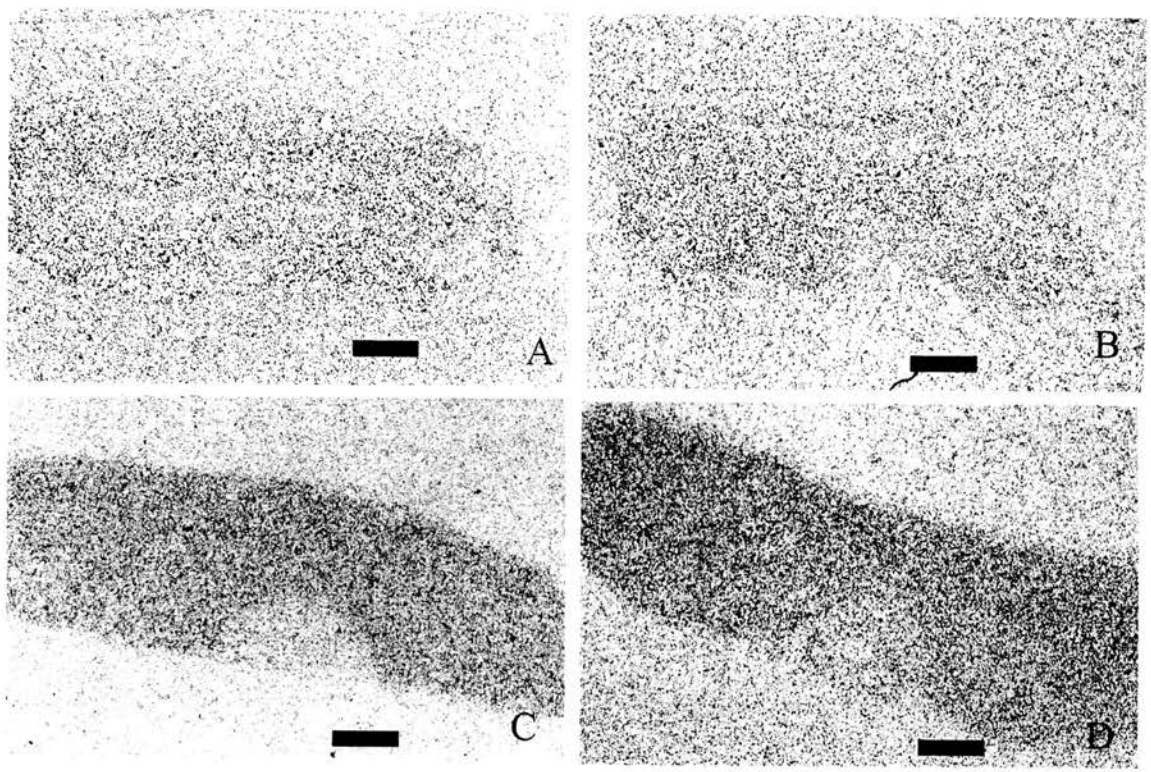


Figure 3.3a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense riboprobes for GR mRNA. A and B were hybridised with labelled sense riboprobe from virgin and day 21 pregnant rats respectively; C and D were hybridised with labelled antisense riboprobe from virgin and pregnant rats respectively. Scale bar = 0.5 mm.

Figure 3.3b Anterior pituitary GR mRNA expression in rats

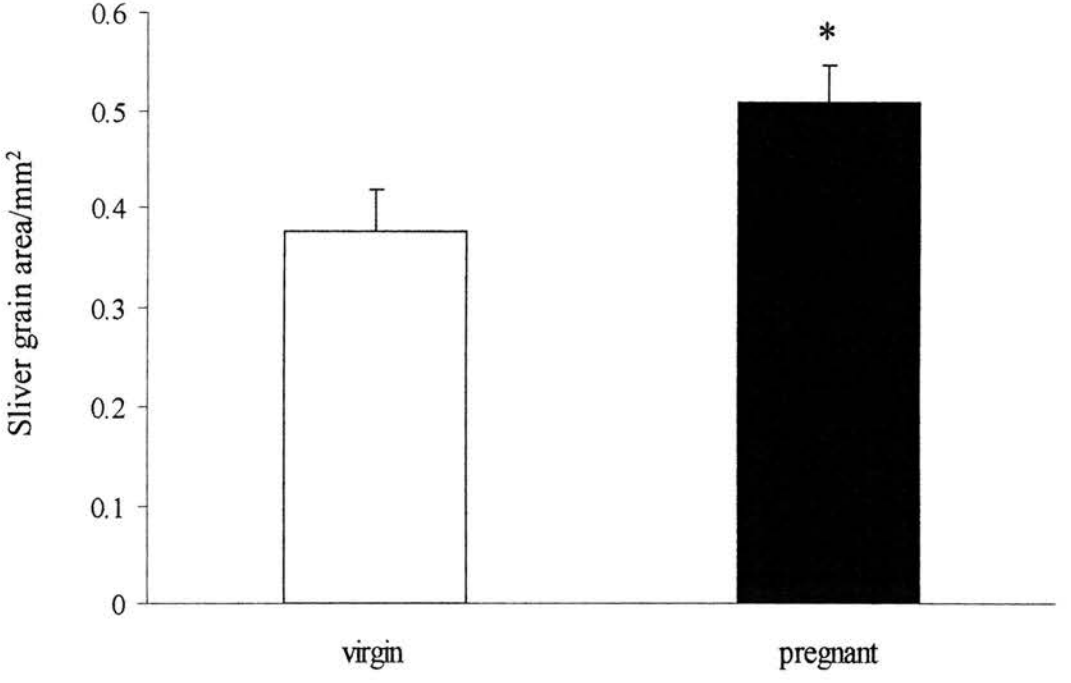


Figure 3.3b. GR mRNA expression in the anterior pituitary collected from virgin and day 21 pregnant rats. Film autoradiographs of pituitary sections probed with a ^{35}S riboprobe for GR mRNA were analysed by a computer-based image analysis system to measure area of silver grains, and the data expressed as mean±SEM silver grain density over a unit area. Anterior pituitary GR mRNA expression in pregnant rats was increased (n=6, 5 for virgin and pregnant respectively. t-test: *$p<0.05$)

POMC mRNA

Figures 3.4a and 3.4b show anterior pituitary POMC mRNA expression in virgin and pregnant rats. Silver grain density was quantified in film autoradiographs of anterior pituitary section hybridised with a radiolabelled oligonucleotide probe. POMC mRNA expression was significantly reduced in day 21 pregnant rats (by 45%, *$p<0.0001$ *vs* virgin, one-way ANOVA; virgin, n=16; pregnant day 10, n=9; pregnant day 21, n=18). As the technique of *in situ* hybridisation with the POMC oligonucleotide probe is well established in our lab, we did not use a sense probe as a control in this study.

Effect of phADX on anterior pituitary POMC mRNA expression

Anterior pituitary POMC mRNA expression in vehicle-treated controls was reduced in pregnancy (Student's t-test, $p<0.05$, but one-way ANOVA on all four groups did not show a significant difference). After 48 h pharmacological adrenalectomy POMC mRNA expression was elevated in both virgin and pregnant rats ($p<0.05$, one-way ANOVA), but the increase was greater in virgin rats (Figures 3.5a and 3.5b).

BK channel mRNA and STREX mRNA

Anterior pituitary BK channel and STREX mRNA expression in the anterior pituitary was evaluated in virgin and pregnant mice. Silver grain density was measured in film autoradiographs of hybridised anterior pituitary sections. Neither BK channel nor STREX mRNA expression was changed in day 18 pregnant mice compared with virgins (Figures 3.6a, 3.6b, 3.7a, 3.7b and 3.8; values are means ±SEM; virgin, n=7; pregnant, n=8).Using oligonucleotides probes, we measured the BK channel and STREX mRNA expression in virgin and pregnant anterior pituitaries in both rat and mouse species. The reason for measuring expression of these two genes expression in mice is because most of the literature on their function is about studies on AtT20 cells, a mouse corticotroph tumor cell line. ACTH secretory responses to stress are reduced in pregnant mice as in pregnant rats (Douglas *et al.* 2001a; Douglas *et al.* 2001b). In the present study, BK channel and STREX mRNA expression in rats and mice showed a similar pattern. Expression of neither was different between virgin and pregnant rats (Figures 3.9a, 3.9b, 3.10a, 3.10b and 3.11; values are means ±SEM; t-test, $p>0.05$; virgin, n=5, pregnant, n=8).

Figure 3.4a Anterior pituitary POMC mRNA expression in rats

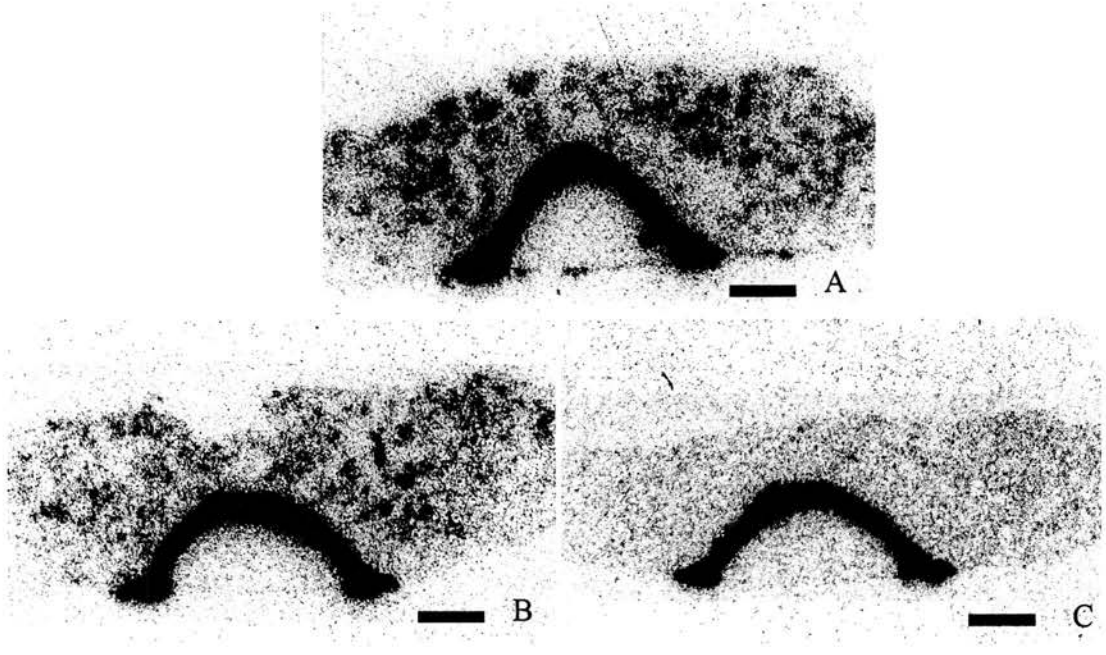


Figure 3.4a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with a ^{35}S- labelled oligonucleotide probe for POMC mRNA. A, B and C were hybridised with labelled POMC probe from virgin, day 10 and day 21 pregnant rats respectively. Scale bar = 0.5 mm.

Figure 3.4b Anterior pituitary POMC mRNA expression in rats

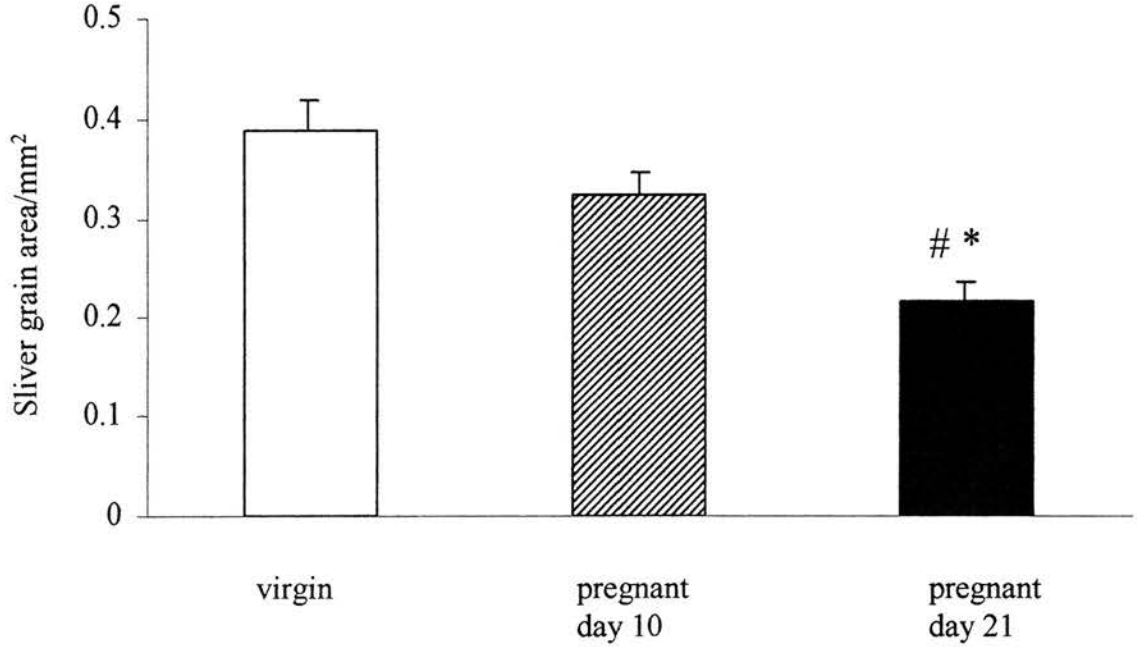


Figure 3.4b. POMC mRNA expression in the anterior pituitary collected from virgin, day 10 and day 21 pregnant rats. Film autoradiographs of pituitary sections probed with a ^{35}S oligonucleotide probe for POMC mRNA were analysed by a computer-based image analysis system to measure area of silver grains, and the data expressed as mean±SEM silver grain density over a unit area. Anterior pituitary POMC mRNA expression in day 21 pregnant rats (n=18) was reduced compared with virgin (n=16, one way ANOVA, *$p<0.001$) and day 10 pregnant rats (n=9, one way ANOVA, #$p<0.001$)

Figure 3.5a PhADX on anterior pituitary POMC mRNA expression in pregnant rats

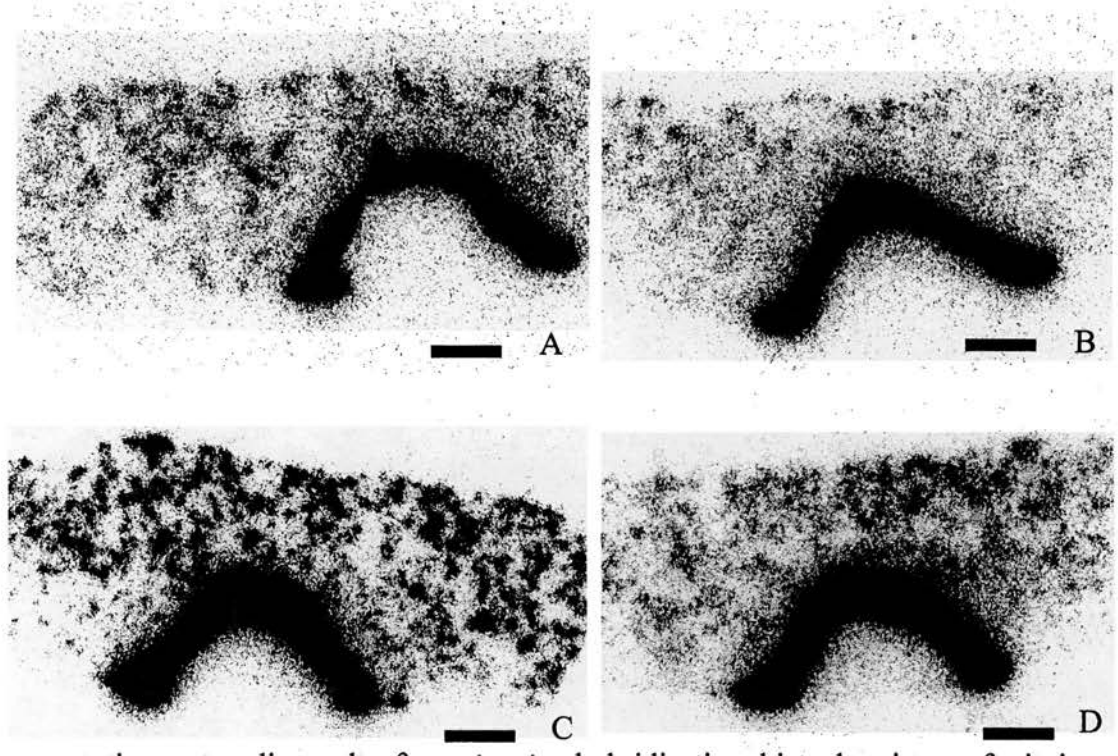


Figure 3.5a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled antisense POMC mRNA. A and B were from vehicle treated virgin and pregnant rat respectively. C and D were from 48 h- phADX virgin and day 21 pregnant rats respectively. Scale bar = 0.5 mm (objective: 5 x).

Figure 3.5b PhADX on anterior pituitary POMC mRNA expression

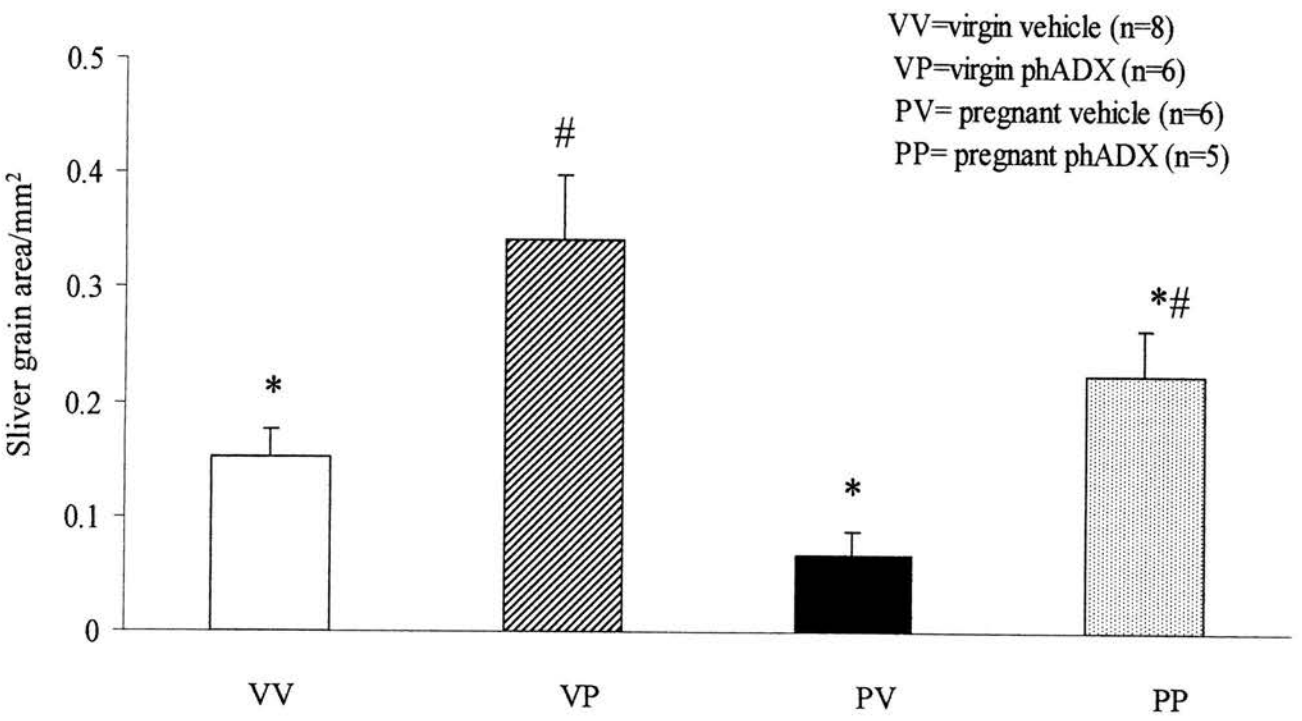


Figure 3.5b. POMC mRNA expression in the anterior pituitary collected from 48 h pharmacological adrenalectomised (phADX) or vehicle treated virgin and day 21 pregnant rats. Film autoradiographs of pituitary sections probed with a ^{35}S riboprobes of POMC mRNA were analysed by a computer-based image analysis system to measure area of silver grains, and the data expressed as mean±SEM silver grain density over a unit area. POMC mRNA expression was reduced in pregnancy (Student's t-test, $p<0.05$, but one-way ANOVA on all four groups did not show a significant difference). After 48 h phADX POMC mRNA expression was elevated in both virgin and pregnant rats ($p<0.05$, one-way ANOVA), but the increase was greater in virgin rats. *$p<0.05$ vs virgin phADX rats; #$p<0.05$ vs respective vehicle-treated rats.

Figure 3.6a Anterior pituitary BK mRNA expression in pregnant mice

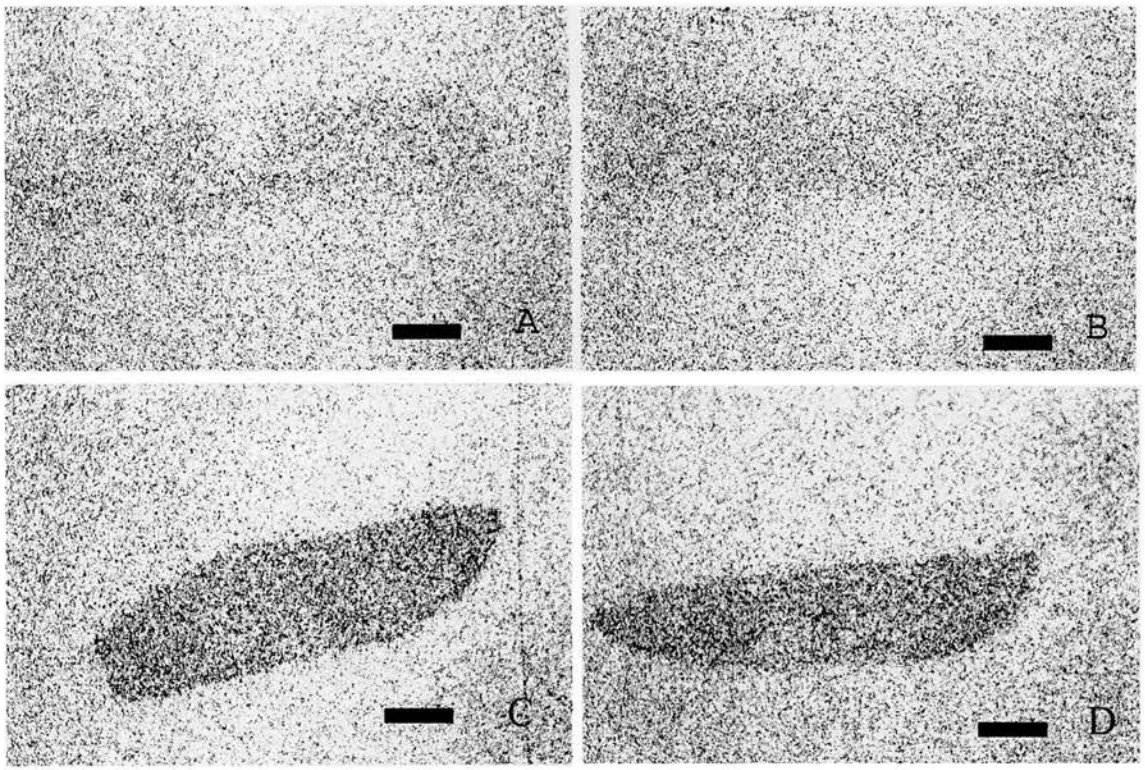


Figure 3.6a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense oligonucleotide probes for BK channel mRNA. A and B were hybridised with labelled sense riboprobe from virgin and day 18 pregnant mice respectively; C and D were hybridised with labelled antisense riboprobe from virgin and day 18 pregnant mice respectively. Scale bar = 0.5 mm (objective: 5 x).

Figure 3.7a Anterior pituitary STREX mRNA expression in pregnant mice

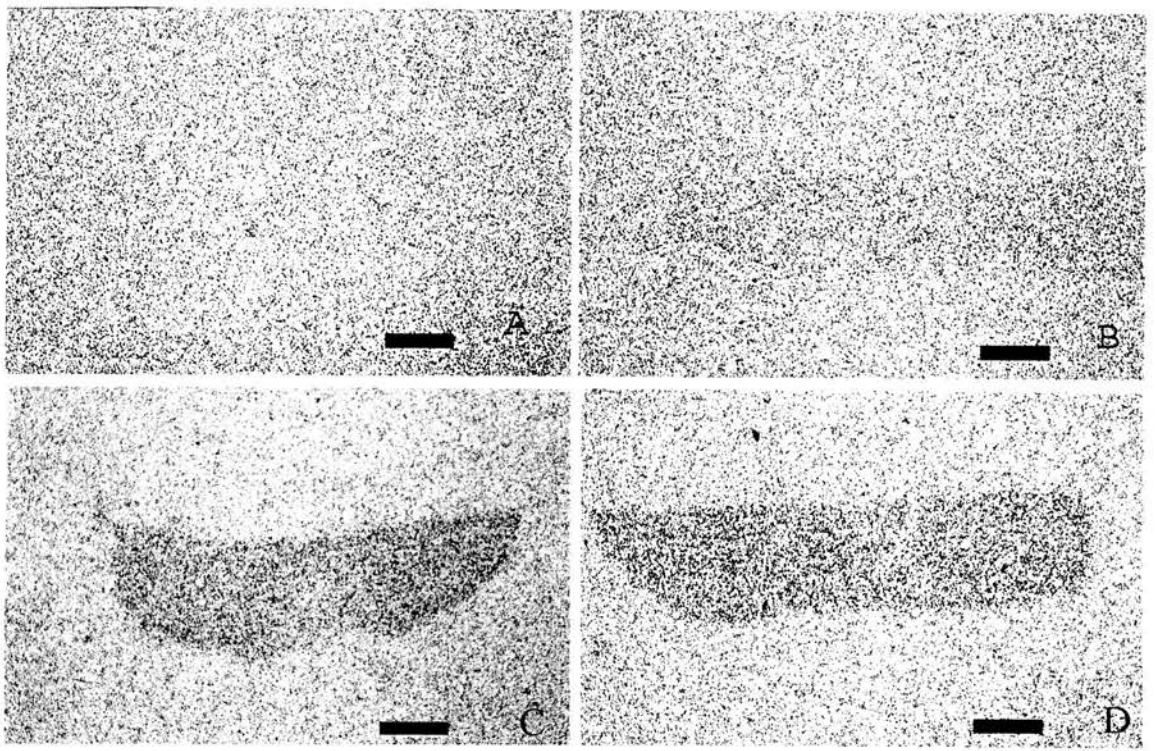


Figure 3.7a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense oligonucleotide probes for STREX mRNA. A and B were hybridised with labelled sense riboprobe from virgin and day 18 pregnant mice respectively; C and D were hybridised with labelled antisense riboprobe from virgin and day 18 pregnant mice respectively. Scale bar = 0.5 mm (objective: 5 x).

Figure 3.6b Anterior pituitary BK mRNA expression in mice

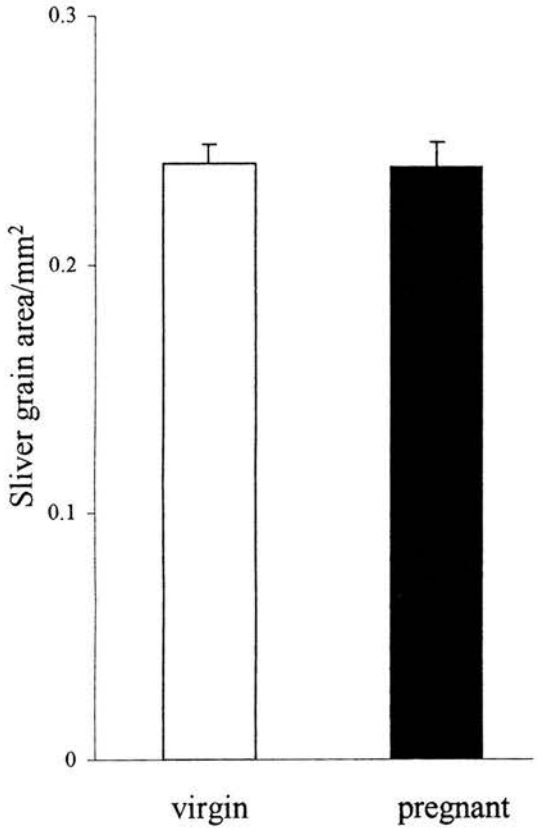


Figure 3.7b Anterior pituitary STREX mRNA expression in mice

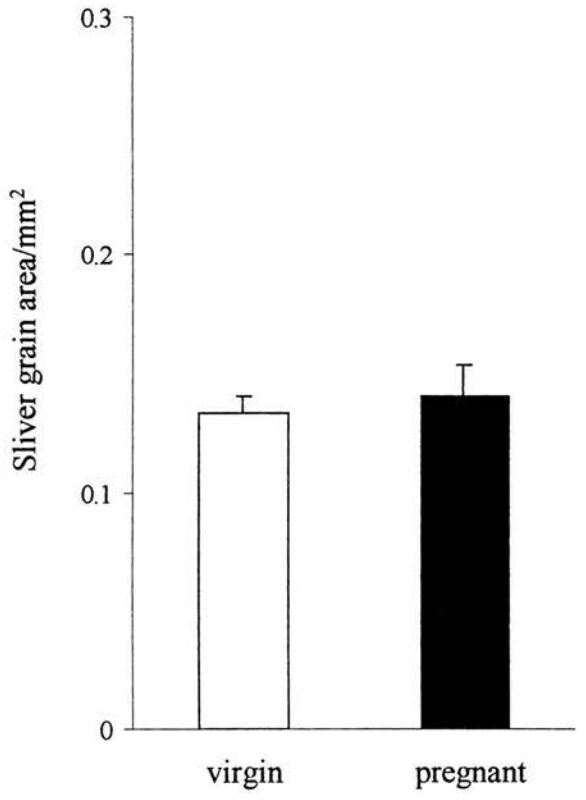


Figure 3.8 Ratio of STREX/BK expression in the mouse anterior pituitary

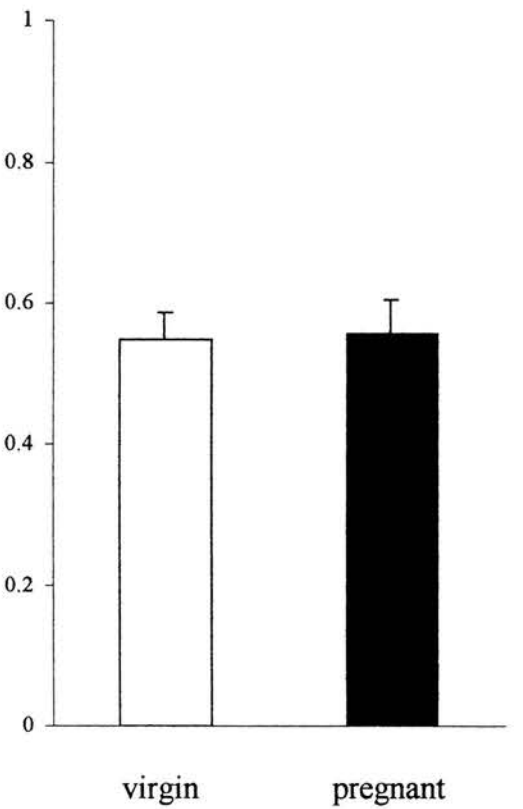


Figure 3.6b, 3.7b, and 3.8 BK mRNA and STREX mRNA expression in the anterior pituitary collected from virgin and day 18 pregnant mice. Film autoradiographs of pituitary sections probed with ^{35}S oligonucelotide probes of BK mRNA and STREX mRNA were analysed by a computer-based image analysis system to measure area of silver grains, and the data data expressed as mean±SEM silver grain density over a unit area. There were no differences in BK or STREX mRNA expression, nor in the ratio of STREX/BK between virgin and pregnant mice (n=7, 8 for virgin and pregnant mice respectively, t-test, $p>0.05$)

Figure 3.9a Anterior pituitary BK mRNA expression in pregnant rats

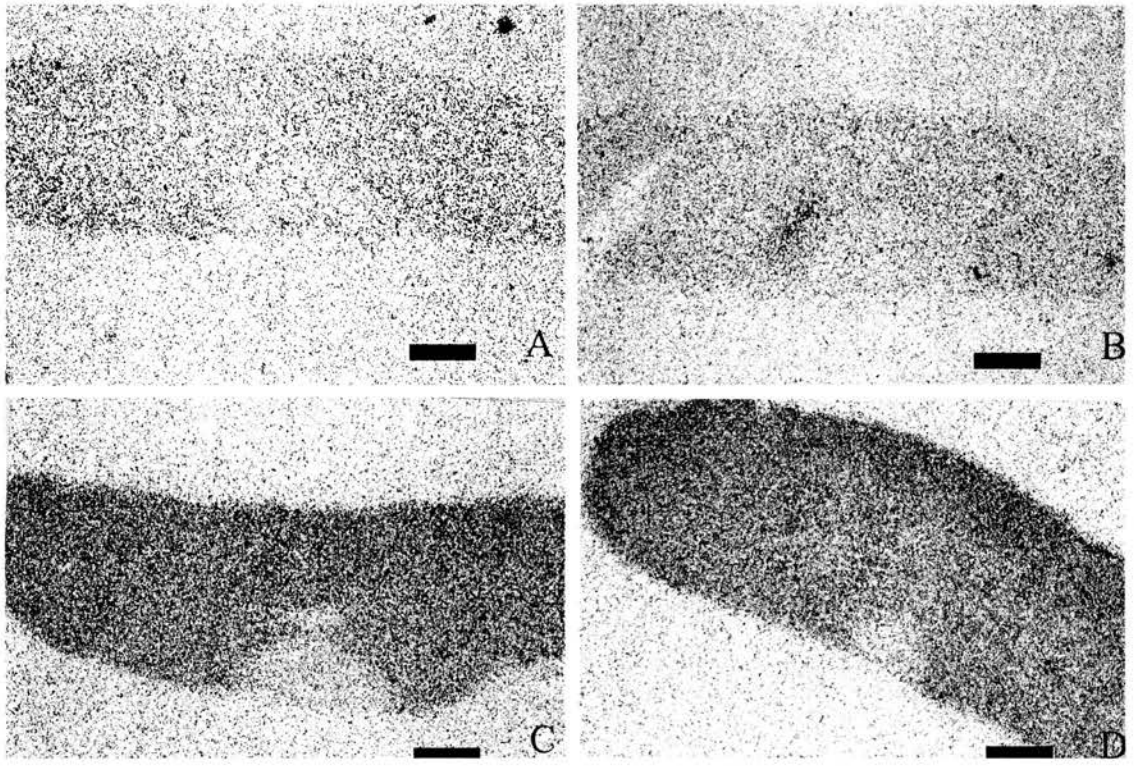


Figure 3.9a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense oligonucleotide probes for BK channel mRNA. A and B were hybridised with labelled sense riboprobe from virgin and day 21 pregnant rats respectively; C and D were hybridised with labelled antisense riboprobe from virgin and day 21 pregnant rats respectively. Scale bar = 0.5 mm (objective: 5 x).

Figure 3.10a Anterior pituitary STREX mRNA expression in pregnant rats

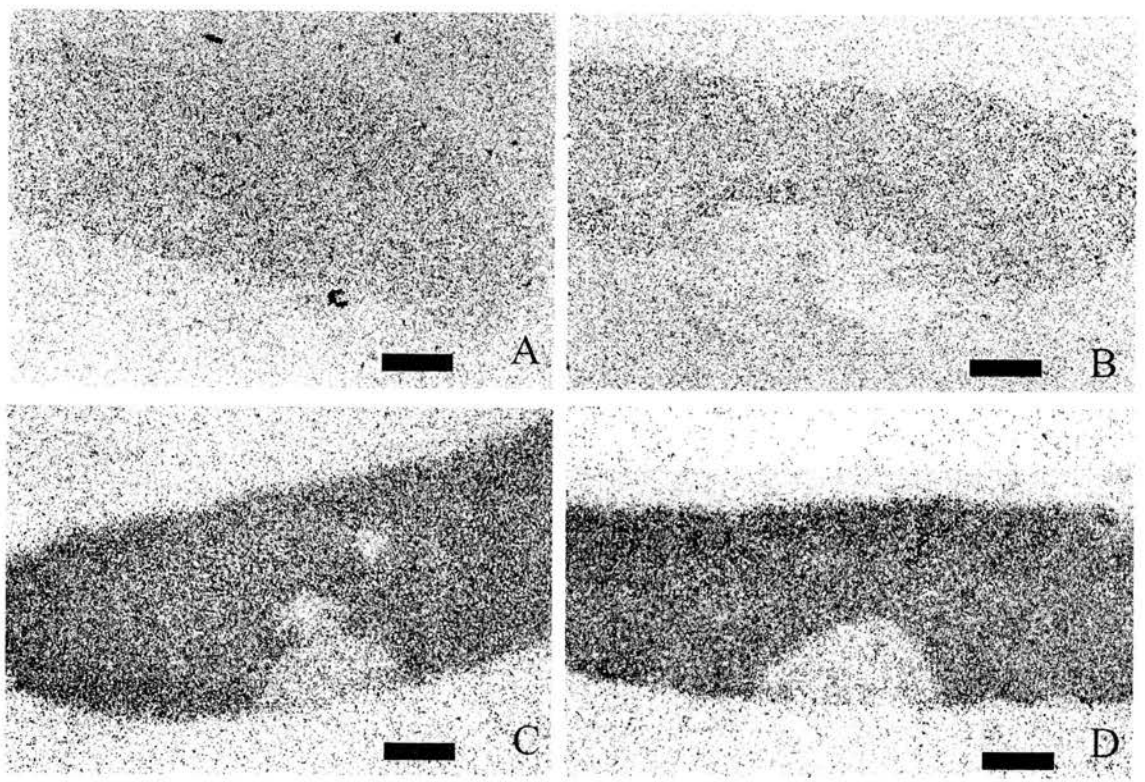


Figure 3.10a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense oligonucleotide probes for STREX mRNA. A and B were hybridised with labelled sense riboprobe from virgin and day 21 pregnant rats respectively; C and D were hybridised with labelled antisense riboprobe from virgin and day 21 pregnant rats respectively. Scale bar = 0.5 mm (objective: 5 x).

Figure 3.9b Anterior pituitary BK mRNA expression in rats

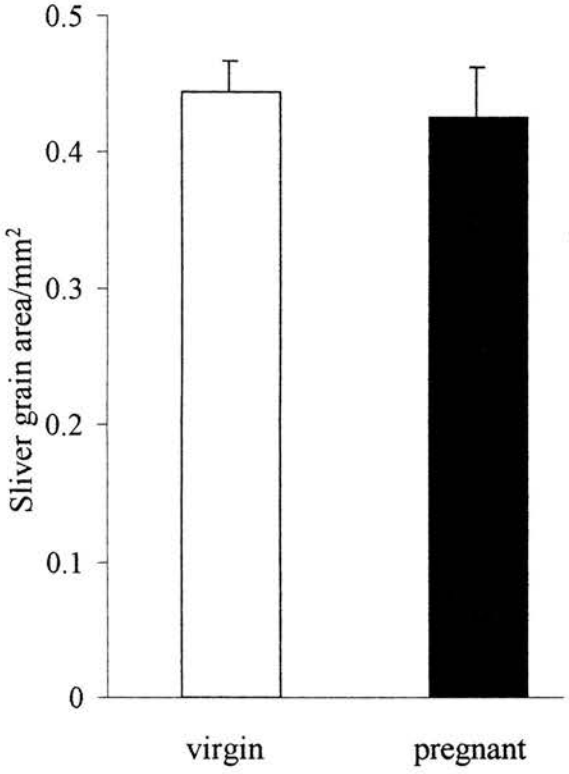


Figure 3.10b Anterior pituitary STREX mRNA expression in rats

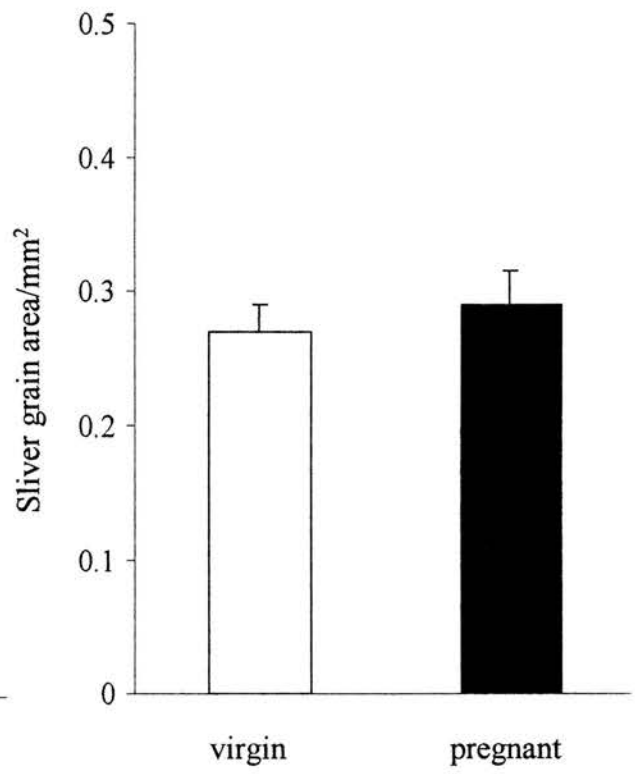


Figure 3.11 Ratio of STREX/BK expression in rat anterior pituitary

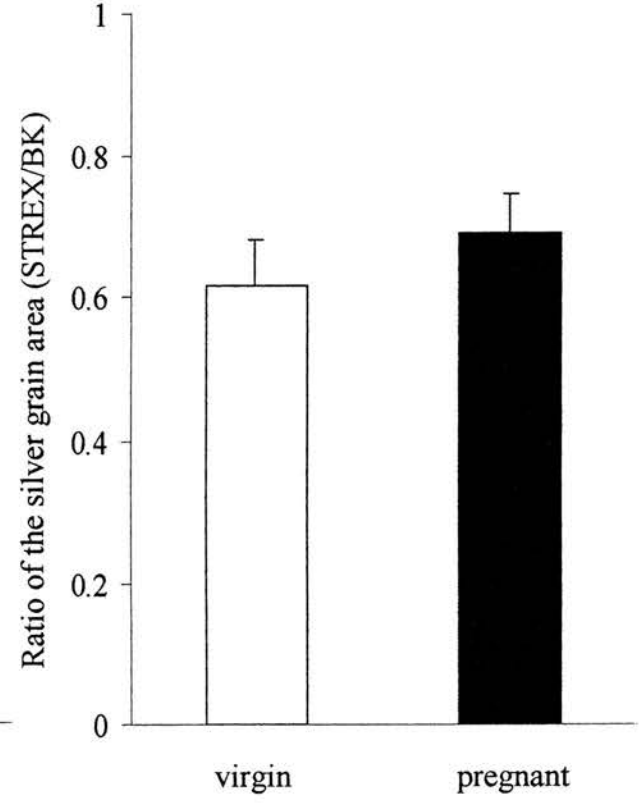


Figure 3.9b, 3.10b, and 3.11 BK mRNA and STREX mRNA expression in the anterior pituitary collected from virgin and day 21 pregnant rats. Film autoradiographs of pituitary sections probed with ^{35}S oligonucleotide probes of BK mRNA and STREX mRNA were analysed by a computer-based image analysis system as the mean area of silver grain and data expressed as mean±SEM silver grain density per unit are of section. There were no differences in BK or STREX mRNA expression, nor in the ratio of STREX/BK between virgin and pregnant rats (n=5, 8 for virgin and pregnant rats respectively, t-test, $p>0.05$)

CRHBP mRNA

Anterior pituitary CRHBP mRNA expression was measured with a 969 bp riboprobe. CRHBP mRNA expression (Figures 3.12a and 3.12b) was found in the anterior pituitary and there was no signal from the sense probe control. There was no difference in CRHBP mRNA expression between virgin and pregnant rat anterior pituitaries (one-way ANOVA, $p>0.05$). One hour after a 30 min restraint stress, CRHBP mRNA was increased in both groups (one-way ANOVA, $p<0.05$), but with no difference between virgin and pregnant rats ($p>0.05$; virgin non stress, n=5; virgin stress, n=8; pregnant non stress, n=5; pregnant stress, n=8).

3.5 Discussion

CRHR1 mRNA expression

Anterior pituitary CRHR1 density measured by [^{125}I] CRH binding is decreased at the late stage of rat pregnancy (Neumann *et al.* 1998). However, CRHR1 mRNA expression in the anterior pituitary in the present study did not change in pregnant rats compared with virgin rats. This inconsistency between receptor and receptor mRNA expression suggests that there is a difference in the post-translation regulation between virgin and pregnant rats. This may be due to the difference in efficiency of mRNA transcription, and/or mRNA and receptor degradation. In this respect, the increased circulating corticosterone concentration in late pregnancy may play a role, because some receptor mRNAs, such as insulin receptor, are more stable in the presence of dexamethasone (Hines *et al.* 1994). The reduced CRH receptor number previously reported may however partially account for the reduced *in vivo* ACTH response to stress or CRH during pregnancy. However, *in vitro*, the ACTH response to CRH is not decreased, but rather increased at low concentration (0.1 nM, see Chapter 6). Hence, decreased CRH receptor binding in pregnancy could reflect increased turnover.

Figure 3.12a Anterior pituitary CRHBP mRNA expression in virgin and pregnant control and stressed rats

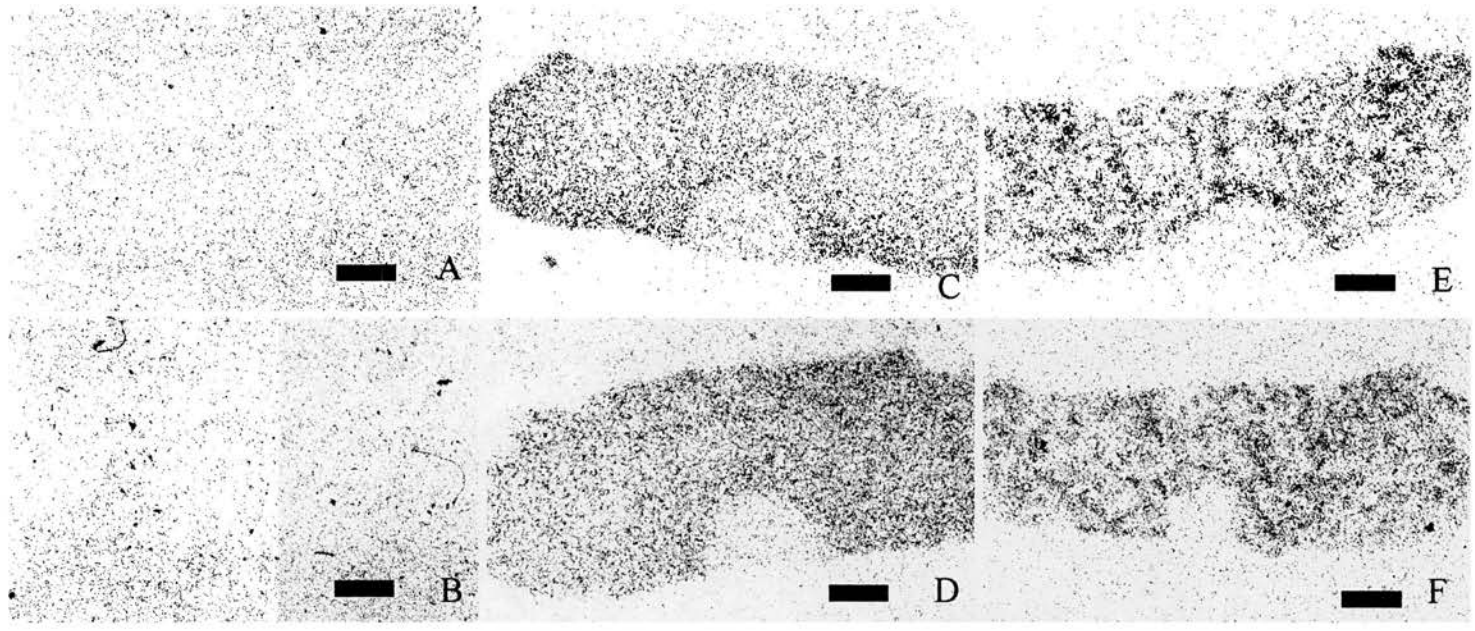


Figure 3.12a Representative autoradiographs from *in situ* hybridisation histochemistry of pituitary sections hybridised with ^{35}S- labelled sense and antisense riboprobe for CRHBP mRNA. A and B were hybridised with labelled sense riboprobe from virgin and pregnant rats respectively; C and D were hybridised with labelled antisense riboprobe from non-stressed virgin and day 21 pregnant rats respectively, E and F were hybridised with labelled antisense riboprobe using tissue collected 1 h after 30 min restraint stress from stressed virgin and day 21 pregnant rats respectively. Scale bar = 0.5 mm (objective: 5 x).

Figure 3.12b Anterior pituitary CRHBP mRNA expression in rats

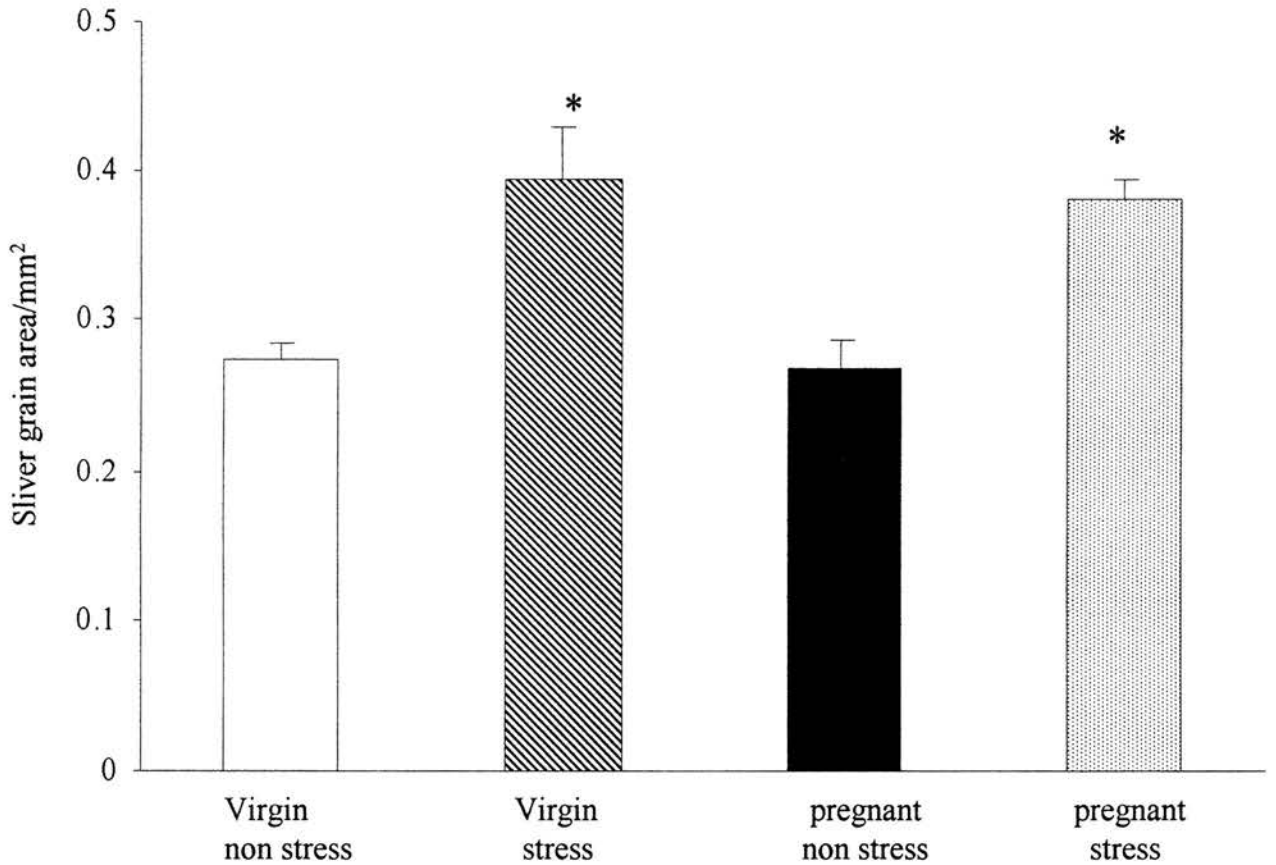


Figure 3.12b. CRHBP mRNA expression in the anterior pituitary collected from virgin and day 21 pregnant rats 1 h after a 30 min restraint stress or no stress. Film autoradiographs of pituitary sections probed with a ^{35}S riboprobes of CRHBP mRNA were analysed by a computer-based image analysis system to measure area of silver grains, and the data expressed as mean±SEM silver grain density over a unit area. The stress increased CRHBP mRNA expression significantly both in virgin and pregnant rats (virgin, n=5, 8 for non stress and stress groups respectively; pregnant, n=5, 8 for non stress and stress groups respectively. *$p<0.05$ *vs* respective non stress groups, One-way ANOVA). There was no difference in CRHBP mRNA expression between virgin and pregnant rats with or without restraint stress.

Previous combined *in situ* hybridisation and immunohistochemistry studies found CRHR1 mRNA signals are located in the intermediate lobe and in subsets of anterior pituitary cells. Although a few non-ACTH reactive cells are CRHR1 mRNA positive, the majority of CRHR1 mRNA positive cells are ACTH reactive (Potter *et al.* 1994).

The CRH receptor is one of the primary targets for negative feedback on the pituitary by glucocorticoids. The administration of dexamethasone causes a decrease in corticotroph CRHR1 mRNA expression either *in vivo* or *in vitro* (Luo *et al.* 1995; Makino *et al.* 1995; Pozzoli *et al.* 1996; Iredale & Duman 1997). Glucocorticoids can inhibit CRHR1 mRNA expression directly (Pozzoli *et al.* 1996; Iredale & Duman 1997) or inhibit CRH and vasopressin expression in the PVN by negative feedback, with a subsequent effect on CRHR1 expression in the corticotrophs. So it is reasonable to consider that the unchanged CRH R1 mRNA at late pregnancy is partially due to the counter-balanced chronic effects of modestly elevated corticosterone concentration in the plasma (Atkinson & Waddell 1995).

CRH is an inhibitor of corticotroph CRHR1 mRNA expression (Pozzoli *et al.* 1996; Rabadan-Diehl *et al.* 1997b). CRH mRNA expression in the PVN is reduced during pregnancy, so it is proposed that portal blood levels of CRH decrease as well (see Chapter 5). The consequent decreased inhibitory effect of CRH on anterior pituitary CRHR1 mRNA expression, together with the prolonged direct inhibitory corticosterone effect, could result in the unchanged CRHR1 mRNA expression in late pregnancy.

The effect of vasopressin on CRHR1 mRNA expression in the anterior pituitary is controversial. *In vitro* studies show vasopressin decreases CRHR1 mRNA expression and augments the downregulatory effect of CRH (Pozzoli *et al.* 1996). However, *in vivo*, chronic administration of vasopressin increases the levels of CRHR1 mRNA in the anterior pituitary (Rabadan-Diehl *et al.* 1996). It is not known what is the possible mechanism(s) of the vasopressin regulatory action on CRHR1 mRNA expression during pregnancy.

The positive correlation between anterior pituitary CRHR1 and POMC mRNA levels in saline and dexamethasone treated male Fischer rats (Zhou *et al.* 1996) was not

found in the present study between the SD strain virgin and pregnant rats. This disruption of the positive correlation suggests that there must be other factors except corticosterone such as sex steroids that contribute to CRHR1 expression regulation (Nappi & Rivest 1995).

V1b receptor mRNA expression

Vasopressin is a weak ACTH secretagogue alone, but augments the effect of CRH significantly. Vasopressin secreted from parvocellular neurones in the PVN is secreted into portal blood from axon terminals in the external zone of the median eminence, is responsible for ACTH stimulation (Antoni 1993). The ACTH stimulation effect of vasopressin is mediated by V1b receptors on the surface of corticotrophs.

V1b receptor is expressed in the majority of pituitary corticotrophs and only a small proportion is in thyrotrophs (Michael *et al.* 1986; Lolait *et al.* 1995). The changes in anterior pituitary vasopressin receptor and V1b receptor mRNAs thus mainly represent those in corticotrophs (Aguilera *et al.* 1994; Rabadan-Diehl *et al.* 1995; Rabadan-Diehl *et al.* 1997a; Rabadan-Diehl & Aguilera 1998).

Toufexis et al have reported that anterior pituitary V1b receptor density is reduced in day 18 or 19 gestation rats in a [^{125}I]-vasopressin binding study (Toufexis *et al.* 1999). To further investigate V1b receptor mRNA expression we used *in situ* hybridisation technique. The results showed that anterior lobe V1b receptor mRNA expression was decreased in pregnancy as well, which is consistent with the receptor density study. Though at the end of pregnancy oxytocin content accumulated in the posterior pituitary increases by 50% (Russell & Leng 1998), oxytocin leakage to the anterior pituitary is supposed to be very low and oxytocin binds only weakly to V1b receptor (Lutz-Bucher B 1983), so it is unlikely that the reduced receptor density is due to oxytocin occupancy. The expression of CRH and vasopressin mRNAs in the parvocellular hypothalamus is reduced during pregnancy (Johnstone *et al.* 2000), so it is apparent that the reduced receptor density in corticotrophs is not due to an increase in occupancy by vasopressin, but to a decrease in receptor synthesis and/or enhanced receptor degradation. The reduced V1b receptor mRNA expression during pregnancy

in the present study supports the former hypothesis. However, how the turnover rate of V1b receptor may be altered is not known.

Vasopressin stimulation of ACTH secretion is resistant to glucocorticoid negative feedback (Abou-Samra *et al.* 1986; Bilezikjian *et al.* 1987). Indeed, Rabadan-Diehl et al. reported glucocorticoid or dexamethasone administration *in vivo* or *in vitro* potentiated vasopressin-stimulated inositol phosphate formation (Rabadan-Diehl & Aguilera 1998). This glucocorticoid potentiation of IP formation is calcium independent and involves increasing cellular levels of GTP binding protein in the pituitary and facilitating V1b receptor coupling with phospholipase C. So the reduced V1b receptor density and mRNA expression in the anterior pituitary may contribute to the attenuated ACTH secretory responses during pregnancy, but the V1b receptor mRNA cannot be a major reason, because corticotrophs have a substantial pool of spare V1b receptor mRNA and the amount of this mRNA is not the primary determinant of receptor content (Rabadan-Diehl *et al.* 1995; Rabadan-Diehl *et al.* 1997a) (see Chapter 6, *in vitro* CRH/vasopressin on ACTH secretion).
It could be that at the late stage of pregnancy, increased corticosterone secretion (Atkinson & Waddell 1995) may increase the production of IP_3 in corticotrophs in response to vasopressin as well, and increase ACTH secretion as a consequence. However, this increase in ACTH secretion may not compensate the possible reduced vasopressin input from the PVN (Chapter 5) and the decreased receptor density on corticotrophs.

Adrenalectomy and dexamethasone replacement studies show glucocorticoids up-regulate V1b receptor mRNA expression (Rabadan-Diehl *et al.* 1997a; Rabadan-Diehl & Aguilera 1998). The increase in corticosterone during pregnancy clearly does not have a net stimulatory effect on V1b receptor mRNA expression in the anterior pituitary, from the present study. The role of endogenous CRH and vasopressin in regulating V1b receptor mRNA expression is not clear. But chronic stress models (repeated immobilisation, chronic hypoglycemia, and chronic psychosocial stress) suggest that vasopressin positively regulates V1b receptor mRNA. Hypothalamic vasopressin mRNA expression in parvocellular CRH neurones and vasopressin, but not CRH, release into the portal blood is increased in these models (de Goeij *et al.* 1991; de Goeij *et al.* 1992a; de Goeij *et al.* 1992b). V1b receptor mRNA expression is

reported to increase following daily immobilisation or intraperitoneal hypertonic saline injection for 8-14 days (Rabadan-Diehl *et al.* 1995). However, acute intraperitoneal hypertonic saline injection causes V1b receptor mRNA expression to decrease, which is prevented by vasopressin antagonist administration (Rabadan-Diehl *et al.* 1995). Since vasopressin is secreted by the hypothalamus into the portal circulation in a pulsatile fashion in the conscious sheep (Engler *et al.* 1989), the divergence between inhibitory and stimulatory vasopressin effects on V1b receptor mRNA expression in chronic and acute models is considered to be the result of different patterns of vasopressin secretion. So the vasopressin secretion pattern may play a role in the reduced V1b receptor mRNA expression, but it is difficult to know the pattern of vasopressin secretion in rats. Decreased V1b receptor mRNA expression may be because of reduced gene transcription, but the possibility cannot be ruled out of increased in-stability of receptor mRNA in the context of changes in corticosterone, regulatory proteins and sex steroids during pregnancy.

GR mRNA expression

GR is expressed in a variety of cells within the anterior pituitary including 99% of the growth hormone and ACTH cells, 67% of TSH cells, 93% of the folliculostellate cells and 94% of the marginal layer cells (Ozawa *et al.* 1999). The measurements made on the anterior pituitary in the present study reflect the overall change of GR expression in these cell types.

Glucocorticoid, the end product of the HPA axis, is an important regulatory factor in the ACTH secretory response. Corticosterone is considered to act on the hippocampus, hypothalamus and pituitary to inhibit the HPA axis system. There are dual receptor mechanisms that mediate this negative feedback. Occupancy studies showed that GRs mediate the negative feedback of elevated glucocorticoid levels to restrain HPA drive, whereas MRs mediate the tonic inhibitory control of the hippocampus on HPA basal activity (de Kloet & Reul 1987; Reul *et al.* 2000).

At the anterior pituitary level corticosterone might influence POMC expression *in vivo* at least at three levels: by alteration of stimulatory CRH input to the corticotroph, by changing CRH receptor responsiveness of the corticotroph itself to CRH input, and

by decreasing expression of the POMC gene. This inhibitory effect of glucocorticoid is mediated by GR located within corticotrophs. Upon glucocorticoid entry into cells, after intranuclear translocation, the GR-GR dimers are constituted. The receptor dimers bind to glucocorticoid response elements (GREs) in the flanking region of target genes and induce transcription of hormone-regulated genes (Trapp *et al.* 1994)

In late pregnancy the elevated plasma corticosterone levels (Atkinson & Waddell 1995) suggest a possible enhanced negative feedback mechanism. The results reported by H A Johnstone et al. show that there is no change in PVN GR mRNA expression, but an increase in dentate gyrus only among the hippocampus subareas across pregnancy. However MR mRNA expression is not altered in the brain in pregnant rats compared to virgins (Johnstone *et al.* 2000). Anterior pituitary GR immunoreactivity in the cytosol measured by a western blot is increased in pregnant ewes (Roesch & Keller-Wood 1999). In the present study, GR mRNA expression in the anterior pituitary was increased near the end of pregnancy. It is not known during pregnancy in which cell type(s) GR mRNA was expressed among the multiple cell phenotypes of the anterior pituitary, including whether GR mRNA expression is increased in corticotrophs. However, increased GR mRNA expression suggests an increase in sensitivity of glucocorticoid negative feedback and a possible enhanced inhibitory role in regulating ACTH secretion during pregnancy. In contrast with the finding in this study, there is no change in GR in the rat pituitary assessed in a [^{3}H]dexamethasone binding study (Meaney *et al.* 1989) during the first two weeks of lactation, which suggest that different regulation exists between the pregnant and lactation states, permitting a sustained elevation in pituitary-adrenal activity during lactation (Stern *et al.* 1973; Lightman 1992).

The mechanism(s) of altered GR expression during pregnancy are not clear. Anterior pituitary GR gene expression is self-regulated by increased glucocorticoid concentrations. Chronic corticosterone or dexamethasone administration increases anterior lobe GR mRNA expression (Sheppard *et al.* 1990). 5 day small dose dexamethasone replacement after adrenalectomy significantly increases GR mRNA content (Sheppard *et al.* 1990). GR mRNA expression is also regulated by CRH. *In vivo* studies showed that adrenalectomy or CRH alone treatment does not change pituitary GR binding capacity (McEwen 1979) or GR mRNA (Sheppard *et al.* 1990),

whereas CRH decreases and dexamethasone increases GR mRNA in adrenalectomised rat pituitary (Sheppard *et al.* 1990). In the AtT20 cell line, CRH administration causes a rapid time-dependent decrease in GR mRNA levels that precedes a dose- and time-dependent decrease in GR binding capacity. Treatment with either forskolin or 8-bromo-cAMP, mimicks the CRH-induced decrease in GR binding, and in addition forskolin decreased GR mRNA levels (Sheppard *et al.* 1991). During pregnancy the decreased CRH mRNA expression (Johnstone *et al.* 2000), reduced CRH content in the median eminence (Chapter 4) and increased basal glucocorticoids (Johnstone *et al.* 2000) may cause the increased GR mRNA expression in the anterior pituitary.

In the past 10 years, several studies suggest that sex steroids influence GR gene expression. In the anterior pituitary, sex-dependent differences in corticosteroid binding were assessed in individual pituitaries from adult male and female rats that had been adrenalectomised 12 h before they were killed. Gonadally intact females showed significantly less GR content than did intact males. When ovariectomised females were compared with gondadectomised males, there was no difference in receptor concentration. Estrogen was able to reverse the effect of ovariectomy: ovariectomised females receiving estrogen had significantly fewer receptors than intact males. Progesterone did not antagonise the effect of estrogen in the pituitary (Turner 1990). Administration of 17β-estradiol completely reverses the ovariectomy-induced increase in GR mRNA content of the pituitary gland (Peiffer & Barden 1987).

Estrogen increases amygdala (Peiffer *et al.* 1991), but decreases hippocampus and pituitary GR mRNA expression (Carey *et al.* 1995; Turner 1990). Hypothalamic GR mRNA levels increase after 17β-estradiol treatment, and this increase is reversed by progesterone (Redei *et al.* 1994). Progesterone can also regulate the binding of glucocorticoids to GR by interacting with the GR binding domain (Roesch & Keller-Wood 1999) to increase the dissociation rate of glucocorticoid from GR (Chou & Luttge 1988).

In pregnant rats plasma progesterone levels are high until the day before partuition, and estrogen level continues to increase. From the above studies, it seems unlikely

that these sex steroids are responsible for increased anterior pituitary GR mRNA expression in pregnancy.

As GR mRNA is not only expressed in the corticotrophs, but also in other cell types in the anterior pituitary (Kononen *et al.* 1993; Ozawa *et al.* 1999), the present study cannot rule out the possibility of increased GR mRNA expression in other cell types, rather than in corticotrophs, during pregnancy.

POMC mRNA expression

POMC mRNA expression is mainly regulated by two ACTH secretagogues, CRH and vasopressin, and by glucocorticoid negative feedback (Bruhn *et al.* 1984). In this study, POMC mRNA cxpression in the anterior pituitary was reduced in day 21 pregnant rats, but not on day 10 of pregnancy compared with virgins. In late pregnancy, portal blood levels of CRH and vasopressin are suggested to be decreased from the *in situ* hybridisation studies that show that PVN CRH and vasopressin gene expression at this time is reduced (Johnstone *et al.* 2000), and plasma glucocorticoid levels are increased (Atkinson & Waddell 1995). The reduced stimulation and increased negative feedback can be at least partly the reason for the reduced POMC mRNA expression in pregnancy.

This presumed decrease in ACTH precursor suggests biosynthesis of ACTH may be reduced in day 21 pregnant rats. However, POMC processing and/or ACTH secretory patterns may change during pregnancy as well. The secretion of ACTH per 24 h near the end of pregnancy is decreased as the afternoon rise in secretion is essentially abolished (Atkinson & Waddell 1995). Furthermore, ACTH content in the anterior pituitary and the secretory capacity are not decreased (see Chapter 6). Chronic immobilisation and Brattleboro rat studies provide some support for changes in processing. Chronic immobilisation increases pituitary POMC mRNA levels, while does not change the basal ACTH levels of adrenalectomised rats, which suggests that chronic stress might also alter POMC processing and/or ACTH secretory patterns in the anterior pituitary in adrenalectomised animals (Marti *et al.* 1999). The results from studies in homozygous (di/di) Brattleboro rats show that vasopressin does not change pituitary POMC mRNA levels, but plasma ACTH and β-endorphin are reduced. This is the evidence that vasopressin may also take a role in POMC biosynthesis, with

decreased degradation or blunted release of POMC products from the pituitary (Canny *et al.* 1988).

PhADX on anterior pituitary POMC mRNA expression

Anterior pituitary POMC mRNA expression in the vehicle treated pregnant rats was less than that in vehicle treated virgin rats, which is consistent with the results above. In late pregnancy, plasma corticosterone levels are increased in rats, which suggests a mechanism of enhanced negative feedback by glucocorticoids may exist. In the present study, after blocking corticosterone production by phADX, POMC mRNA expression was elevated significantly in both virgin and pregnant groups. This indicates that POMC mRNA expression in the anterior pituitary is under tonic inhibition by glucocorticoids in both virgin and pregnant rats. Indeed, it is consistent with the plasma ACTH concentration changes after phADX, where ACTH concentrations were increased from about 9 pg/ml in both virgin and pregnant vehicle-treated rats to about 65 pg/ml in phADX virgin rats and to only about 30 pg/ml in pregnant rats (PJ Brunton, unpublished, personal communication; measured in the same groups of rats). Plasma corticosterone concentrations were greater in vehicle treated pregnant rats (about 120 ng/ml) compared with vehicle treated virgin rats (about 82 ng/ml); 48 h phADX did not block plasma corticosterone production completely with still substantial levels of corticosterone in plasma (about 50 ng/ml and 35 ng/ml in virgins and pregnant rats respectively (PJ Brunton, unpublished, personal communication; measured in the same groups of rats). Pharmacological adrenalectomy caused a greater reduction of corticosterone concentration in pregnant rats (by 32 ng/ml and 85 ng/ml in virgin and pregnant rats respectively). However, the increases in POMC mRNA expression were about the same in virgin and pregnant rats after phADX (ca. 0.19 and 0.16 silver grain density mm^2/mm^2, for virgin and pregnant rats respectively). As plasma corticosterone concentration was lower in the phADX pregnant rats than in the phADX virgin rats, this suggests, together with the similar increases in POMC mRNA expression, that sensitivity of POMC mRNA expression to glucocorticoid feedback inhibition is greater in pregnancy. Furthermore, the greater elevation of plasma ACTH concentrations in phADX virgin than in phADX pregnant rats also indicates greater slow feedback sensitivity of ACTH synthesis and secretion to glucocorticoid in pregnancy. The increased anterior

pituitary GR mRNA expression in pregnancy described above may underlie enhanced glucocorticoid negative feedback in anterior pituitary corticotrophs in pregnancy.

BK channel mRNA and STREX mRNA

The release of ACTH from corticotrophs is largely dependent on stimulation of Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels (Nishizuka 1984; Luini *et al.* 1985; Won *et al.* 1990; Guerineau *et al.* 1991). The CRH-induced membrane depolarisation is due to inhibition of BK-channel by PKA. The inhibition of BK channels leads to increased intracellular calcium levels, with the consequence of ACTH release. A splice variant of the BK channel α-unit, STREX, is the functional unit existing in about 90% of BK channels (Shipston *et al.* 1999). In the present study, we did not find a different expression of BK channel or STREX mRNA in the anterior pituitary in pregnancy. These results suggest that the contributions of BK channels to the regulation of corticotrophs are not different between virgin and pregnant.

CRHBP mRNA expression

In this study, CRHBP mRNA was detected in the anterior pituitary which is consistent with a previous report (Potter *et al.* 1992). One hour after a 30 min restraint, CRHBP mRNA expression in the anterior pituitary was increased 50% compared with the non-stress groups in both virgin and pregnant rats. Previous studies show that CRHBP gene expression in the anterior pituitary is positively regulated by CRH, cAMP, stress, and glucocorticoids (Woods *et al.* 1994; Cortright *et al.* 1997; McClennen *et al.* 1998). The mechanism of stress-induced elevated CRHBP mRNA expression is not known. It may be due to the high levels of CRH, cAMP and glucocorticoids after stress. However, we did not measure any difference in CRHBP mRNA expression between virgin and pregnant rats before or after restraint stress. So even if CRHBP is a regulator of the corticotroph response to stimulation by CRH, it may not be an important factor in the attenuated ACTH response to stress during pregnancy.

The lack of a difference in the increase in CRHBP mRNA expression in the anterior pituitary after stress between virgin and pregnant rats, in contrast with the attenuated secretion of ACTH in pregnant rats, may reflect regulation of CRHBP mRNA expression by glucocorticoid. The corticosterone response to restraint stress in pregnancy is reduced, but it is attenuated much less than the ACTH response

(Neumann *et al.* 1998). Hence, stimulation of CRHBP mRNA expression by glucocorticoid may be similar in pregnant and virgin rats. CRH release may not be changed in pregnant rats compared to in virgins after stress (Chapter 4), and cAMP effect may be enhanced in pregnancy as suggested from secretion studies (Chapter 6). All together, these factors may contribute to no changes in CRHBP mRNA expression in the anterior pituitary.

Summary: in anterior pituitary glands from pregnant rats, there was no change in CRHR1 gene expression, but decreased V1b receptor mRNA expression. The latter corresponds to a previously reported decrease in V1b receptor binding; but a previous study found decreased CRH receptor binding. Together these changes might lead to reduced effectiveness of CRH and vasopressin (see Chapters 4 and 5). No change of BK channel and STREX mRNA expression suggests no change in the role of these channels in regulating ACTH secretion. Reduced POMC mRNA expression probably reflects reduced stimulation of ACTH secretion. Elevated GR gene expression indicates an enhanced glucocorticoid negative feedback in the pregnant rats. Expression of the mRNA for CRHBP, another factor indirectly inhibiting ACTH secretion, and its stimulation by stress, are similar in pregnant rats as in the virgin ones.

Chapter 4

Role of CRH in ACTH secretion during pregnancy *in vivo*

4.1 Introduction

CRH is the major stimulatory regulator of pituitary ACTH secretion in rodents and its effect on ACTH secretion is potentiated several times by vasopressin (Antoni 1986; Gillies *et al.* 1982). CRH is synthesised in the parvocellular neurones in PVN of the hypothalamus and is transported in the axons of these neurones projecting to the median eminence and then is released into the portal vessels to regulate ACTH secretion from the anterior pituitary (King & Baertschi 1990). CRH as a secretagogue of ACTH secretion from corticotrophs acts through specific plasma membrane receptors, CRHR1. CRHR1 exists in corticotrophs and in discrete brain areas including the cerebral cortex, several regions related to the limbic system and peripheral tissues (Baigent & Lowry 2000; De Souza *et al.* 1985; Millan *et al.* 1987).

During pregnancy, the HPA axis responses to physical and emotional stress are attenuated (Neumann *et al.* 1998). The effectiveness of exogenous CRH on ACTH and corticosterone secretion is also reduced (Neumann *et al.* 1998). The sensitivity of corticotrophs during pregnancy may be changed as suggested from receptor autoradiography studies: CRH receptor binding in the anterior pituitary is decreased in pregnant rats compared with virgins (Neumann *et al.* 1998; Toufexis *et al.* 1999). Lower basal CRH mRNA expression in the PVN of pregnant rats suggests reduced CRH availability for driving the secretion of ACTH (Johnstone *et al.* 2000).

To investigate the effect of CRH on ACTH secretion *in vivo* during pregnancy, we prevented its action by administrating a non-peptide CRHR1 antagonist, N-butyl-N-ethyl-[2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidine-4-yl]-amine, antalarmin, to rats. Antalarmin is a pyrrolopyrimidine compound which has a high affinity for the CRHR1 (Bornstein *et al.* 1998). It blocks CRH-stimulated ACTH release (Webster *et al.* 1996) by acting on corticotroph CRHR1. Antalarmin also acts

in the brain to delay parturition: fetuses infused with vehicle were delivered at a mean gestational age of 141.8 ± 0.9 days compared with antalarmin-infused sheep at 148.8 ± 1.6 days (Chan *et al.* 1998). Behavioural responses are also modified by antalarmin: in rhesus macaques, antalarmin inhibits the behaviours associated with anxiety and fear such as body tremors, grimacing, teeth gnashing, urination, and defecation and increases exploratory and sexual behaviours that are normally suppressed during stress (Habib *et al.* 2000) and in rats, antalarmin impairs CRH-mediated the induction or expression of conditioned fear by cold condition, and also impairs the enhanced fear induced by exposing to inescapable shock (Deak *et al.* 1999). The release of CRH from the hypothalamus may be changed during pregnancy, so CRH contents in the median eminence may be crucial for CRH concentrations in the portal blood.

4.2 Aims

The studies in this chapter were to elucidate the roles of CRH in the attenuated ACTH response to stress during pregnancy: whether reduced CRH release into the portal blood from the hypothalamus may be the underlying mechanism of attenuated ACTH responses to stress. CRH contents in the median eminence and hypothalamus were measured to evaluate the stores of CRH in pregnancy.

4.3 Materials and methods

CRHR1 antagonist and stress-stimulated ACTH secretion

To study CRH action on ACTH secretion, individually caged pregnant and virgin animals had a silastic jugular cannula implanted by the method as described in Chapter 2.1.2.1. The experiment was carried out four to five days following surgery to allow the animal to recover. On the day of experiment (day 21 of pregnancy), the jugular cannula was flushed and connected to a syringe filled with sterile heparinised saline (at 0800 h). Antalarmin (20 mg/kg, a kind gift from Dr. Manolis Zoumakis, NIH, USA) or vehicle (1 ml/kg) was injected intraperitoneally at 08.30- 09.00 h; the vehicle was 10% ethanol and 10% Cremaphor EL in double distilled water.

Antalarmin was dissolved at 60°C and kept at this temperature. Two basal blood samples (0.4 ml) were taken 60 min and 90 min after intraperitoneal injection. All the rats were forced to swim for 90 s in deep water (19°C), and further blood samples were taken after 5, 15 and 70 min. Each blood sample was collected into a tube containing 15 µl 5% EDTA per 100 µl blood. Blood was replaced with 0.9% saline. Blood samples were cooled on ice, centrifuged at 12,000 g for 5 min and plasma separated and stored at -70°C until assayed.

ACTH stimulation by exogenous CRH

These rats were from the experiment in which the CRHR1 antagonist was used. 70 min after stress, all rats were injected with CRH 20 ng/kg (Neumann *et al.* 1998) intravenously and blood samples were collected 10 min later. Immediately another CRH (200 ng/kg) dose (Toufexis *et al.* 1999) was given and blood samples were collected 10 min later. Each blood sample was collected into a tube containing 15 µl 5% EDTA per 100 µl blood. Blood was replaced with 0.9% saline. Blood samples were cooled on ice, centrifuged at 12,000 g for 5 min and plasma separated and stored at -70°C until assayed.

At the end of all of the experiments, the animals were killed by an intravenous overdose of anaesthetic (Sagatal, 36 mg in 600 µl per rat) and pregnancy status checked. ACTH concentrations were assayed with commercially available ACTH kits from ICN, for details see Chapter (details in Chapter 2.2.2.5).

Median eminence and hypothalamic CRH content measurement

Pregnant and virgin animals were caged singly five days before use with food and water available *ad libitum*. On the morning of the experiment (day 10 or day 21 of pregnancy), immediately after the rat was decapitated, the median eminence and the hypothalamus were dissected under a binocular microscope. The brain was placed on a glass slide and the median eminence, with the attached pituitary stalk was separated using microscissors. A block containing the hypothalamus was then prepared with a scalpel blade (coronal cuts just anterior to the optic chiasma and caudal to the mammillary bodies; sagittal cuts along the hypothalamic sulci, and a horizontal cut just ventral to the thalamus). The median eminence and the hypothalamic blocks were snap-frozen on dry ice, and later sonicated in a solution of 0.5 M acetic acid and 0.1

M HCl. The median eminence contents were sonicated in 50 µl solution and the hypothalamus contents in 250 µl. The sonicants were sent by airfreight on dry ice to Prof. Pierluigi Navarra (Catholic University Medical School, Italy) for CRH radioimmunoassay. The study on day 10 pregnant and virgin contents was several months before the study on day 21 pregnant and virgin contents, but CRH measurements were made in the same radioimmunoassay (Details are described in previous reports (Dello *et al.* 2000; Navarra *et al.* 1991), with intra- and interassay coefficients of variation of 5% and 10% respectively). CRH contents per median eminence or hypothalamus were calculated by multiplying the radioimmunoassayed CRH concentration by the respective sonication solution volume. The total hypothalamic CRH content was calculated by adding CRH content of the median eminence and that of the cognate hypothalamus together.

Statistics

Data from the study of CRH effects and of the CRHR1 antagonist on the ACTH response to stress were analysed with two-way ANOVA for repeated measurements followed by post-hoc Student-Newman-Keuls tests. Data about CRH content in the hypothalamus were analysed with t-tests.

4.4 Results

Effect of CRHR1 antagonist, antalarmin, on the ACTH response to stress

To investigate the role of CRH in the ACTH response to stress in virgin and pregnant rats, we suppressed CRH action by an intraperitoneal injection of a CRHR1 antagonist, antalarmin. One and half hours after antalarmin administration, the basal ACTH concentrations before swim stress did not differ between groups (Figure 4.1, $p>0.05$). Following swim stress the ACTH plasma concentrations were significantly increased in all groups at 5 and 15 min ($p<0.05$). Plasma ACTH concentrations returned to close to the basal levels by 70 min post swim stress. The peak ACTH response occurred at 5 min after the stress in all groups ($p<0.05$). Plasma ACTH concentration was lower in response to swim stress in vehicle- treated pregnant rats

compared to vehicle- treated virgins ($p<0.05$) 5 min after swim stress. Antalarmin decreased plasma ACTH levels after swimming in both virgin and pregnant groups compared to the respective vehicle- treated groups at 5 and 15 min after swim stress ($p<0.05$). 70 min after swimming stress, there was no difference in plasma ACTH concentrations between the pregnant and virgin vehicle or antalarmin- treated groups, although the vehicle- and antalarmin- treated pregnant groups tended to be higher ($p>0.05$).

The histograms (Figure 4.2) present the changes (delta) in plasma ACTH concentrations at 5 min and 15 min post swim stress relative to the pre-swim mean basal concentrations. The increments were less in vehicle-treated pregnant rats than in the respective vehicle-treated virgin groups. Antalarmin attenuated the ACTH increments to swim stress by about 55- 57% in both virgin and pregnant rats (virgin vehicle, n=8; virgin antalarmin, n=5; pregnant vehicle, n=9; pregnant antalarmin, n=10) at 5 min, and also decreased ACTH increments at 15 min.

CRH on ACTH response secretion in vivo

All groups were intravenously injected CRH 20 ng/kg 70 min after the swim stress. Ten min after this dose, plasma ACTH concentrations in all groups tended to increase, but did not significantly differ with respect to 70 min basal levels nor among each other (Figure 4.3). Ten min after a further injection of a larger dose of CRH (200 ng/kg), plasma ACTH concentrations increased significantly in all groups. The increases in ACTH concentrations of vehicle-treated virgin rats were significantly higher than those in any other groups ($p<0.05$, two-way ANOVA for repeated measurements) (Figure 4.3). There were no significant differences in plasma ACTH concentrations between vehicle- and antalarmin-treated pregnant groups.

Figure 4.1 Effect of antalarmin on ACTH responses to stress in pregnancy

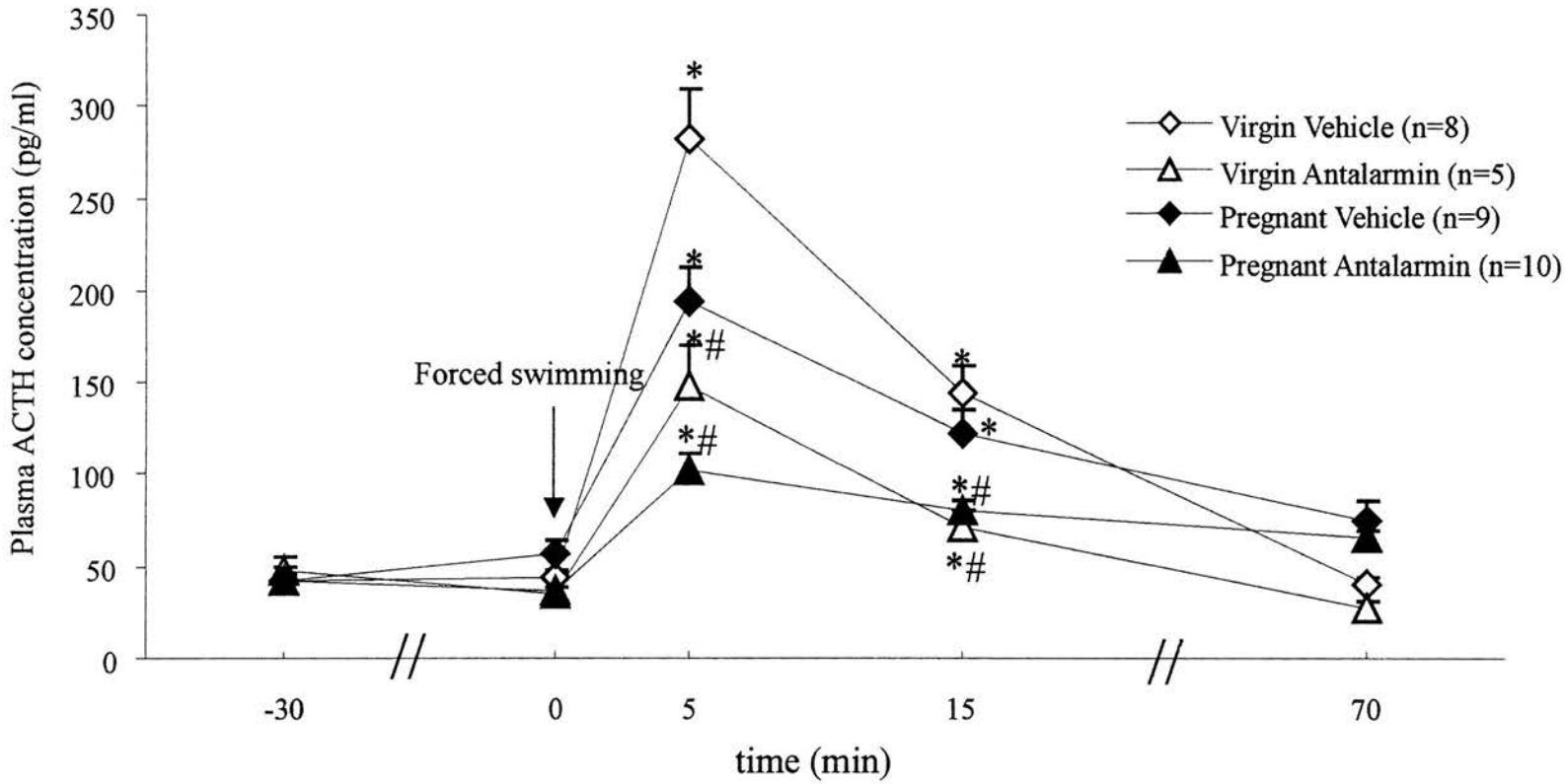


Figure 4.1 Effect of CRHR1 antagonist, antalarmin, on ACTH secretion. Data are means ± SEM. 90 min after antalarmin (20 mg/kg, i.p.) or vehicle injection and the collection of two basal blood samples, all rats were forced to swim for 90 s. Statistical analysis: significant effect of swimming stress at 5 and 15 min ($p< 0.0001$, two-way ANOVA repeated measurements), of pregnancy ($p<0.05$) at 5 min. Antalarmin reduced the ACTH response to forced swimming in both virgin and day 21 pregnant rats at 5 min and 15 min ($p<0.05$, two-way ANOVA repeated measurements followed by post-hoc Student-Newman-Keuls tests. *$p<0.05$ *vs* respective basal, #$p<0.05$ *vs* respective vehicle treated group; @$p<0.05$ *vs* virgin vehicle group.

Figure 4.2 Increases in ACTH concentrations after forced swimming in pregnancy

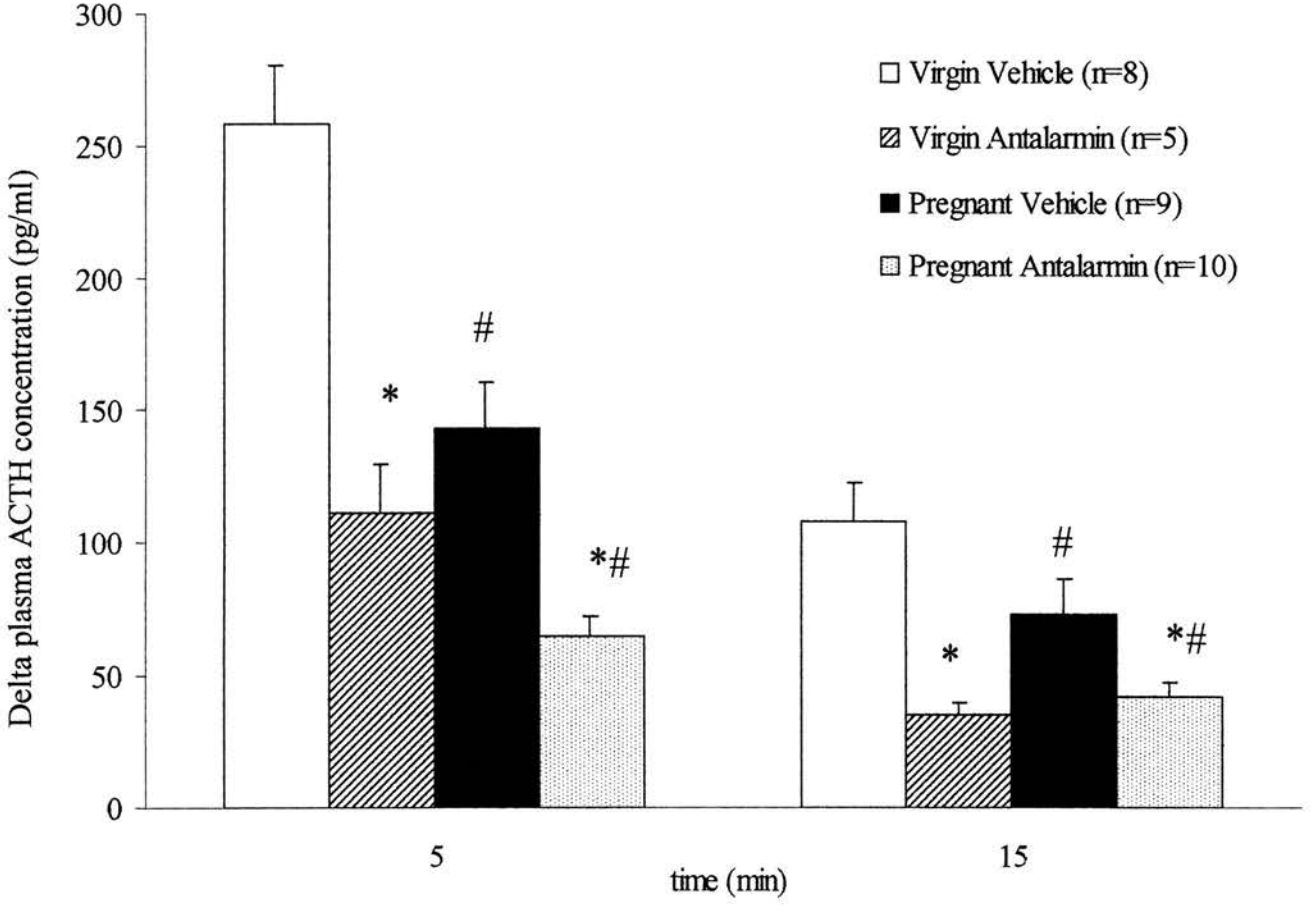


Figure 4.2. Effect of CRHR1 antagonist, antalarmin, on stress-induced ACTH secretion *in vivo*. Data are means±SEM. 90 min after antalarmin (20 mg/kg, i.p.) or vehicle injection and the collection of two basal blood samples, all rats were forced to swim for 90 s. The histograms show differences between mean basal and 5 and 15 min post-swim plasma ACTH concentrations. 5 min post-swim, antalarmin treatment decreased the ACTH increment in response to swimming stress by 56.8% in the virgin rats, and similarly, decreased the increment by 55.1% in the day 21 pregnant rats. *p< 0.05 *vs* respective vehicle, #p<0.05 *vs* virgin vehicle.

Figure 4.3 CRH stimulated ACTH secretion in pregnancy

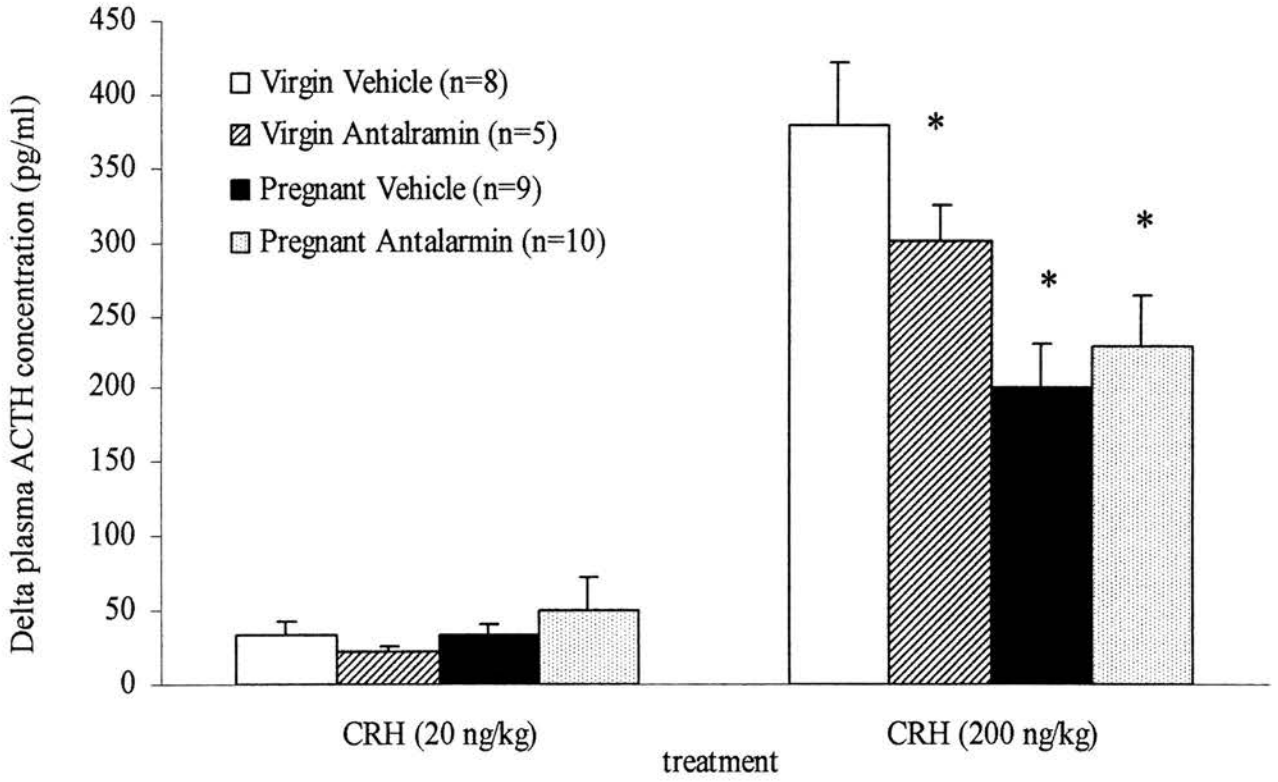


Figure 4.3. Effect of CRHR1 antagonist, antalarmin, on CRH stimulation of ACTH secretion *in vivo*. Data are mean increases from 70 min basal±SEM. 90 min after antalarmin (20 mg/kg, i.p.) or vehicle injection and the collection of two basal blood samples, all rats were forced to swim for 90 s. 70 min after stress, all rats were injected with CRH 20 ng/kg intravenously and blood samples were collected 10 min later. Immediately another CRH (200 ng/kg) dose was given and blood samples were collected 10 min later. ACTH secretion in response to the low dose of CRH (20 ng/kg) was not different between any groups. The high dose CRH (200 ng/kg) treatment increased ACTH secretion more in the vehicle treated virgin group than in any other groups. *$p<0.05$ *vs* virgin vehicle, two-way ANOVA for repeated measurements followed by Student-Newman-Keuls tests.

Hypothalamic and median eminence CRH content

The contents of CRH in the median eminence and in the hypothalamus are presented in Figure 4.4-7 and summarised in Table 4.1-2. CRH content was about 4-8 times greater in the median eminence than in the hypothalamus. CRH content in the median eminence was reduced in pregnancy both on day 10 and day 21 compared with virgins (t-test, $p<0.05$). There was a tendency for CRH content in the hypothalamus of both pregnant groups (day 10 and day 21) to be greater than in virgins but this was not significant (t-test, $p=0.33$, 0.17 respectively). Compared to virgins, the total CRH contents in the whole hypothalamus of both the day 10 and day 21 pregnant groups were not changed. CRH content in the median eminence expressed as a percentage of that in the total hypothalamus, representing the proportion of total CRH available for secretion, was reduced significantly only in the day 21 pregnant group (t-test, $p<0.05$), but not in the day 10 group.

Figure 4.4a CRH content in the median eminence in virgin and day 10 pregnant rats

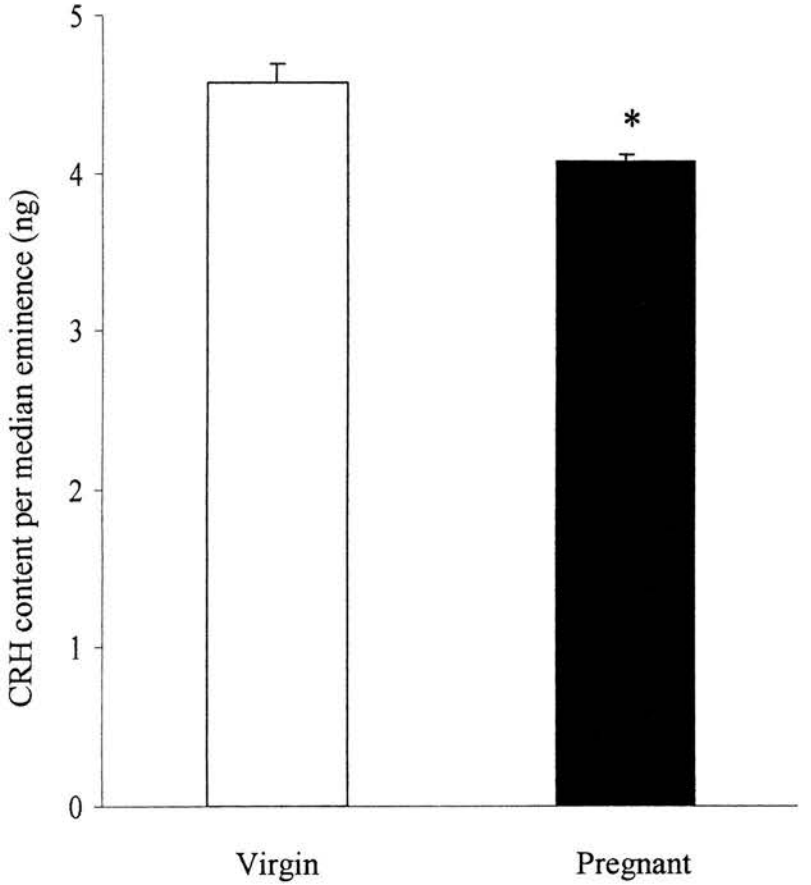


Figure 4.4b CRH content in the hypothalamus in virgin and day 10 pregnant rats

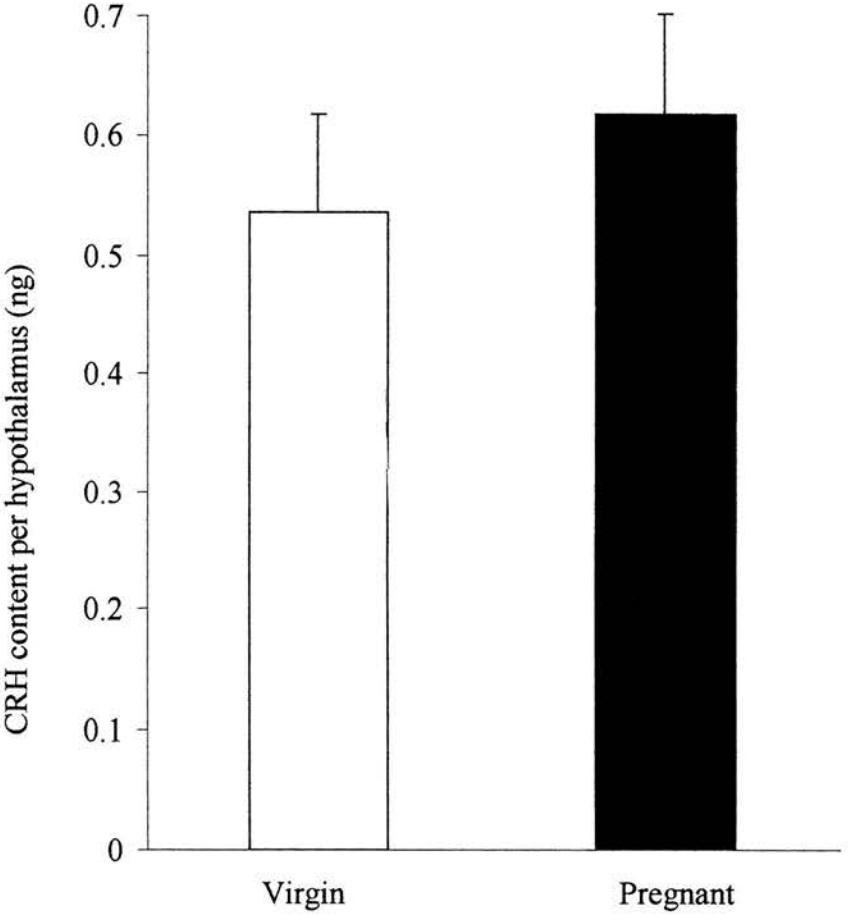


Figure 4.4a, b. Radioimmunoassay determination of CRH contents in the median eminence and the rest of the hypothalamus of virgin and pregnant (day 10 of gestation) rats. Values are presented as mean±SEM. CRH contents in the median eminence of pregnant rats were less than those of virgin rats (*$p<0.05$ *vs* virgin, t-test). CRH contents in the hypothalamus were not significant greater in pregnant rats ($p=0.33$, t-test). Virgin (n=16), pregnant (n=14).

Figure 4.5a CRH content in the median eminence in virgin and day 21 pregnant rats

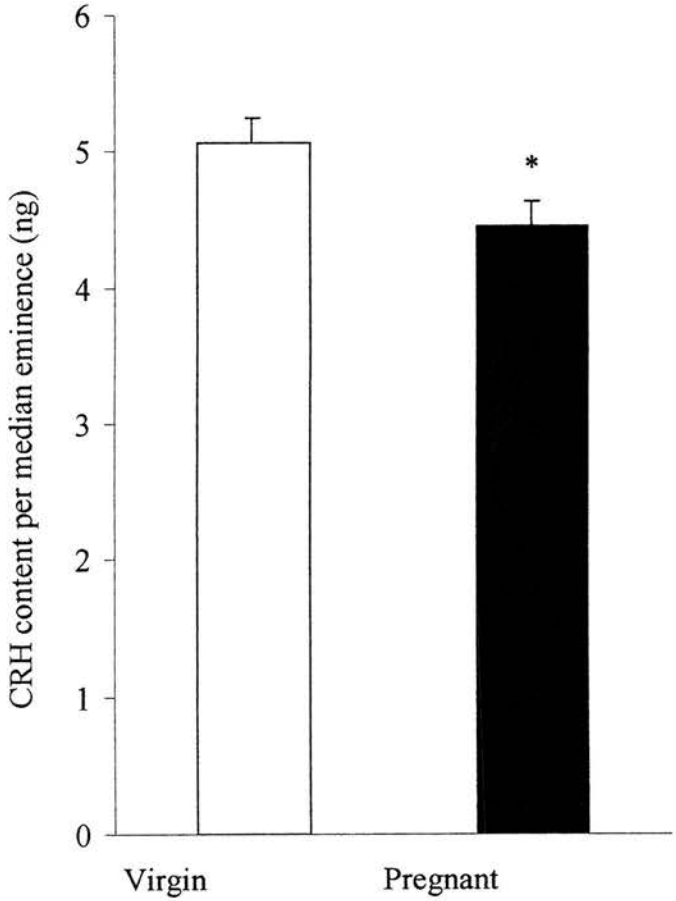


Figure 4.5b CRH content in the hypothalamus in virgin and day 21 pregnant rats

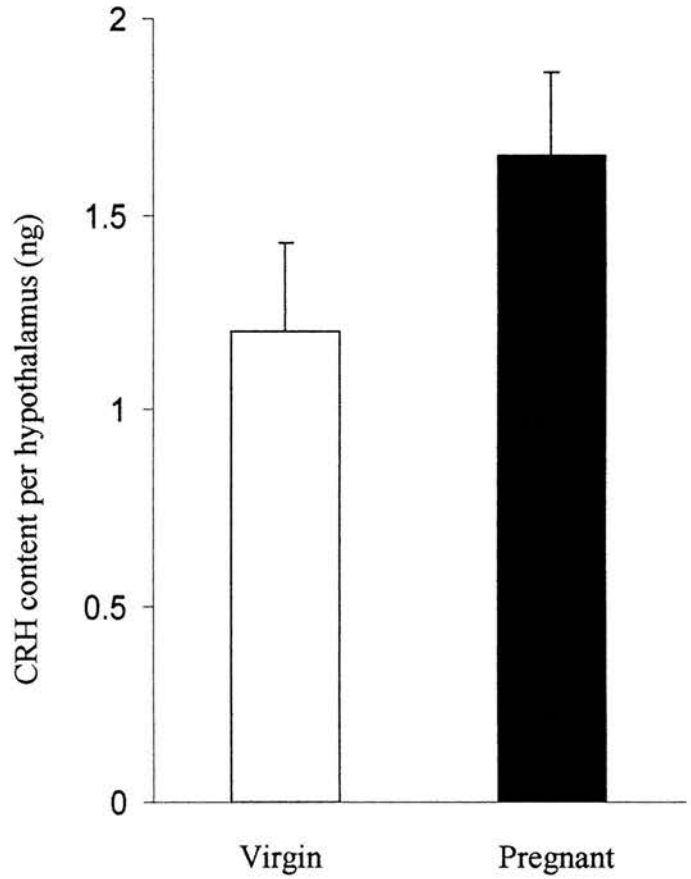


Figure 4.5a, b. Radioimmunoassay determination of CRH contents in the median eminence and the hypothalamus of virgin and pregnant (day 21 gestation) rats. Values are presented as mean±SEM. CRH contents in the median eminence of pregnant rats were less than those of virgin rats (*$p<0.05$ *vs* virgin, t-test). CRH content in the rest of hypothalamus was not significant higher in pregnant rats ($p=0.17$, t-test). Virgin (n=10), pregnant (n=15).

Figure 4.6a CRH content in the whole hypothalamus of virgin and day 10 pregnant rats

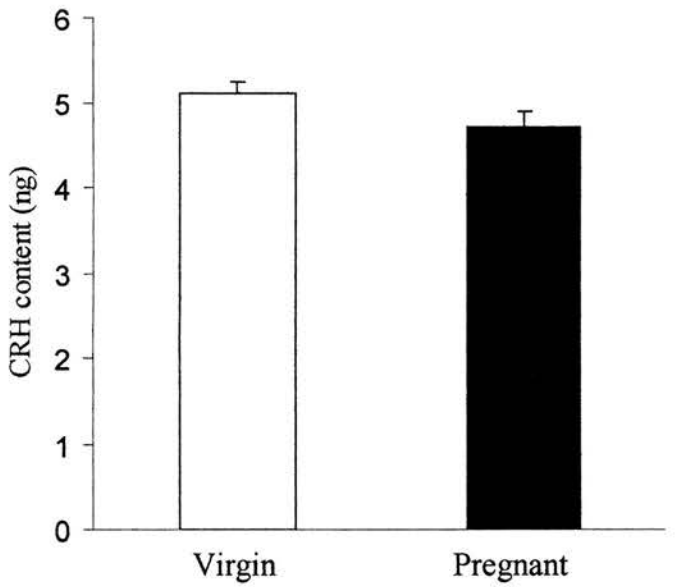


Figure 4.6b CRH content in the whole hypothalamus of virgin and day 21 pregnant rats

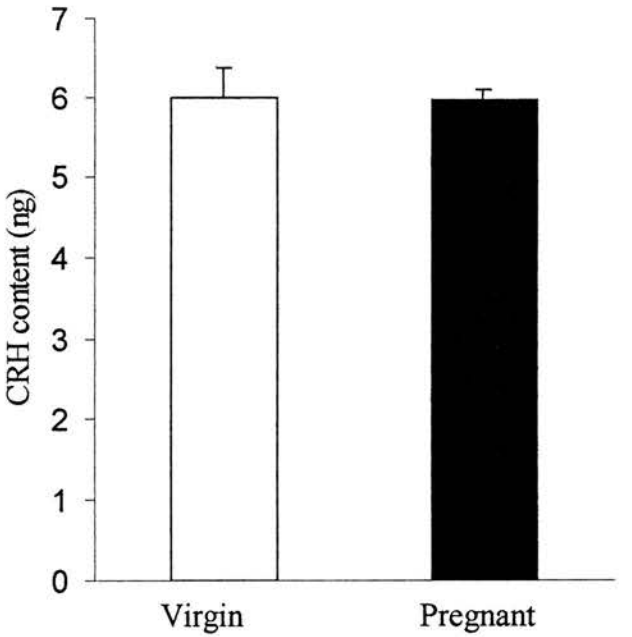


Figure 4.6a, b. The calculated total CRH contents of the whole hypothalamus. The total content of the whole hypothalamus was obtained by adding respective contents of the hypothalamus and the median eminence. There were no differences between virgin and pregnant rats in any group (t-test). For day 10 group: virgin (n=16), pregnant (n=14); for day 21 group: virgin (n=10), pregnant (n=15).

Figure 4.7a Median eminence CRH content as a percentage of that in the whole hypothalamus of virgin and day 10 pregnant rats

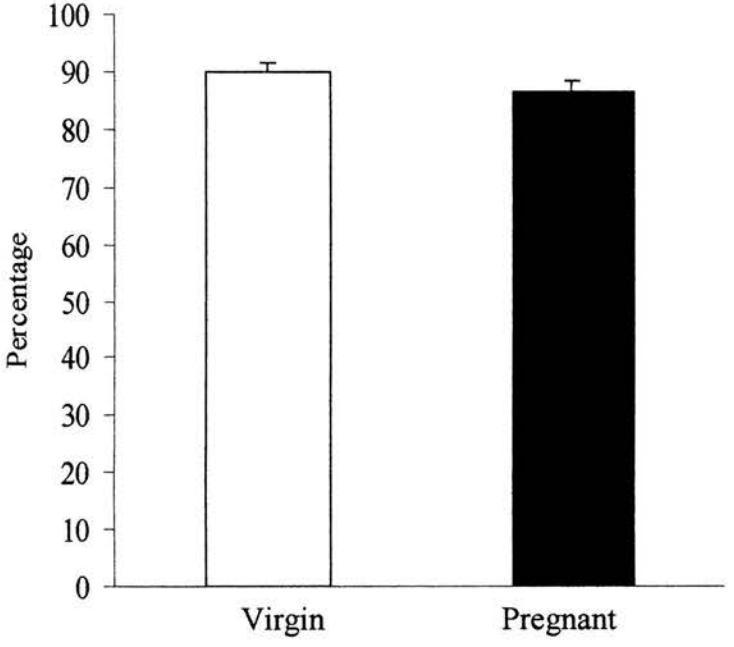


Figure 4.7b Median eminence CRH content as a percentage of that in the whole hypothalamus of virgin and day 21 pregnant rats

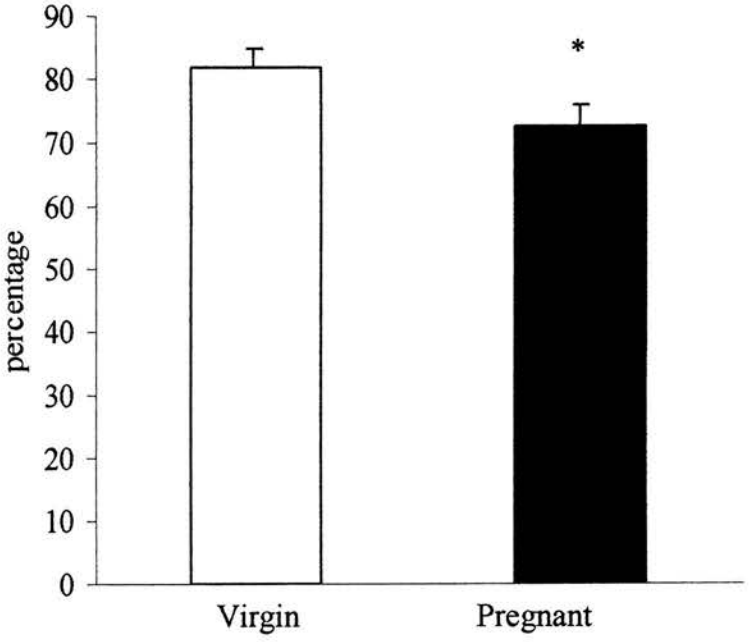


Figure 4.7a, b. Median eminence CRH content as a percentage of that in the respective whole hypothalamus.There was no difference between the virgin and day 10 pregnant groups, but median eminence content was significantly less in the day 21 pregnant group compared with the virgin rats (*$p<0.05$ *vs* virgin, t-test). For day 10 group: virgin (n=16), pregnant (n=14); for day 21 group: virgin (n=10), pregnant (n=15).

Table 4.1. CRH contents in median eminence and the whole hypothalamus in the virgin and day 10 pregnant rats

	Virgin rats n=16		Pregnant rats (day 10) n=14	
	Median eminence	Hypothalamus	Median eminence	Hypothalamus
CRH content (ng)	4.58±0.12	0.54±0.08	4.08±0.05*	0.62±0.08
Total content (ng)	5.11± 0.14		4.70±0.20	
Median eminence content as % of total CRH content	89.83±1.68%		86.67±1.75%	

Table 4.2. CRH contents in median eminence and the whole hypothalamus in the virgin and day 21 pregnant rats

	Virgin rats, n=10		Pregnant rats (day 21), n=15	
	Median eminence	Hypothalamus	Median eminence	Hypothalamus
CRH content (ng)	5.06±0.18	1.20±0.23	4.46±0.18*	1.65±0.21
Total content (ng)	6.00±0.37		5.97±0.12	
Median eminence content as % of total CRH content	81.87±2.88%		72.56±3.22%*	

Table 4.1 and 4.2 These are the summaries of the CRH content measurements from virgin, day 10 and day 21 pregnant rats. Values are means ± SEM, *p<0.05 *vs* virgin, t-test.

4.5 Discussion

Stress or exogenous CRH and ACTH secretion

Results from this investigation demonstrate that pregnancy in rats causes a reduction in the release of ACTH in response to exogenous CRH and swim stress. This is consistent with the previously reported attenuated ACTH response to CRH and swim stress in pregnancy (Neumann *et al.* 1998). Although there is no study to investigate the clearance rate of ACTH during pregnancy so far, it is reported that ACTH clearance rate is not altered by sex steroids during the estrous cycle (Viau & Meaney 1991), and it is unlikely that the attenuated ACTH response is due to an increase in ACTH degradation during pregnancy in the context of the fast secretion time course (within 5 min) after swimming stress or exogenous secretagogue.

In the present study, we applied exogenous CRH (200 ng/kg) at a dose that mimicked the ACTH secretory response to stress. In response to exogenous CRH, the magnitude of the plasma ACTH response was attenuated in late pregnancy compared to virgins. This phenomenon suggests a decrease in pituitary sensitivity to CRH and/or a change in the levels of CRH and vasopressin in the portal blood secreted in response to stress during pregnancy. The endogenous CRH and vasopressin concentrations in the portal blood in pregnancy are not known yet, however, CRH and vasopressin mRNA expression in the PVN of pregnant rats is reduced, as shown with by *in situ* hybridisation (Johnstone *et al.* 2000). So the reduced ACTH secretion in response to exogenous CRH in pregnancy can be deduced to be at least partly due to the interaction with reduced endogenous vasopressin.

The plasma volume is increased in pregnancy by 50% (Nadel *et al.* 1988), but whether and how the distribution of secreted or injected CRH changes in pregnancy is not known. The dose of CRH in this study was given by body weight, so it is unlikely that the reduced ACTH response is due to the different blood drug concentrations (also see Chapter 5).

CRHR1 antagonist and ACTH secretion

CRHR1 contents measured by binding are reduced in the anterior pituitary of pregnant compared with virgin rats (Neumann *et al.* 1998). This suggests the sensitivity of corticotrophs to CRH might change during pregnancy. Furthermore, the basal and CRH-stimulated cAMP levels in pituitaries from pregnant rats are lower than those from virgins (Neumann *et al.* 1998), and this also supports the possibility of reduced sensitivity of corticotroph cells during pregnancy. But the results in this study showed that the CRHR1 antagonist, antalarmin, decreased ACTH increments 5 min after swimming stress similarly in virgin and pregnant rats by about 55-57% of the respective vehicle-treated groups, indicating there is no reduction in CRH effectiveness in pregnancy. Rather, action of the augmenting secretagogue, vasopressin, may be decreased in pregnancy (see Chapter 5)

As there is only one report of antalarmin pharmakokinetics in the case of intravenous and oral administration (Habib *et al.* 2000), but not intraperitoneal administration, the elimination half-life of antalarmin in the blood in the present study is not known. The timing of antalarmin injection and the stress exposure (90 min after antalarmin) in the present study took into account previous tests on behaviour (Deak *et al.* 1999). In virgins, exogenous CRH (200 ng/kg) was more effective in the vehicle-treated group compared with the group given antalarmin 3 h previously. This indicates antalarmin acts directly on CRHR1 on the corticotrophs in the anterior pituitary to block ACTH secretion. However, we cannot explain why antalarmin was not effective in reducing the ACTH response to CRH in pregnant rats at that time (Figure 4.3). It may be due to differences in antalarmin degradation between virgin and pregnant rats, or different sensitivities and time courses of brain actions. As antalarmin is a non-peptide CRH receptor antagonist that can pass through the blood-brain barrier, so its effect in attenuating the ACTH response to stress can reflect both its central and peripheral actions. In the brain, CRHR1 is localised widely in the structures of the brain, such as primate neocortex, amygdala, hippocampus, hypothalamus, and in the locus coeruleus (Chalmers *et al.* 1995; Primus *et al.* 1997). It is possible that antalarmin acts on the PVN in the hypothalamus directly and/or on other brain structures indirectly inhibiting stimulation of stressor- processing and hence CRH secretion.

CRH stores

The hypothalamus contains small stores of CRH under basal conditions (Lightman 1992), and changes in CRH mRNA expression are considered to be a reliable index of CRH secretion (Harbuz & Lightman 1989; Harbuz *et al.* 1990). The results from *in situ* hybridisation show (Johnstone *et al.* 2000) that CRH mRNA expression is decreased in the hypothalamus at the late stage of rat pregnancy, which supports the hypothesis of reduced exposure of the anterior pituitary to CRH from the hypothalamus during pregnancy. As CRH is the major ACTH secretagogue, it is of interest to know the changes in the production of CRH peptide in the hypothalamus and its transport to the median eminence, and then into portal blood during pregnancy. It is very difficult to measure stress-induced ACTH secretagogue concentrations in the portal blood in rats. In this study, we measured the hypothalamus and median eminence CRH contents from the decapitated virgin and pregnant rats to see if reduced store of CRH might contribute to reduced ACTH secretion in pregnancy. CRH content in the hypothalamus tended to be higher in pregnancy, but not significantly. In pregnancy, CRH content was about 10% lower in the median eminence than in virgins with no change in the total hypothalamus content in pregnancy. This suggests that the production of CRH might not be altered, but that transported from the hypothalamus to the median eminence is decreased in pregnant rats. However, the changes in content and distribution of CRH in the hypothalamus in pregnancy may also reflect decreased production, as indicated by decreased parvocellular PVN CRH mRNA content (Johnstone *et al.* 2000). Decreased CRH daily release is also suggested by loss of the evening increase in ACTH secretion (Atkinson & Waddell 1995): basal morning plasma ACTH concentrations do not change during pregnancy, but there is a loss of the diurnal increase in the afternoon. This decrease in CRH in the median eminence may lead to a decrease in CRH release and portal blood concentration after stress during pregnancy. However, in day 10 and day 21 pregnant rats, CRH contents in the median eminence were 87% in day 10 (90% in virgin controls) and 72% in day 21 pregnant rats (82% in virgin controls) of that in the whole hypothalamus respectively. Compared with respective virgin groups, there was a reduction of this percentage by about 3% in day 10 or by 9% in day 21 pregnant rats. This modest decrease in CRH in the median eminence suggests that there is still substantial CRH available for release in the median eminence during stress in pregnancy. In the present study, we can't exclude the possibility that an

increase in CRH release may contribute to the reduced CRH contents in the median eminence in day 21 pregnant rats.

In summary, in the present study, as determined from the actions of a CRHR1 antagonist, CRH contributed similarly to stress-stimulated ACTH secretion in pregnant and virgin rats. However, CRH available for stimulating ACTH secretion in the median eminence was lower in pregnancy, although this may reflect reduced CRH production because of reduced secretion under basal condition in the pregnant rats.

Chapter 5

Effect of vasopressin and the interaction of CRH/vasopressin on ACTH secretion *in vivo*

5.1 Introduction

Vasopressin produced in the parvocellular CRH neurones of the PVN is transported via nerve fibres to the external zone of the median eminence and released into the capillaries of the portal system is responsible for stimulating ACTH secretion (Antoni 1986; Antoni *et al.* 1990; Holmes *et al.* 1986). The action of vasopressin on corticotrophs is mediated through plasma membrane V1b receptor (Jard *et al.* 1986). Vasopressin is co-localised in the parvocellular neurones of the PVN of the hypothalamus with CRH (Whitnall *et al.* 1985; Makara 1992). The proportion of vasopressin-positive neurones increases following adrenalectomy, acute and chronic stress paradigms associated with hypersensitivity of the HPA axis (Whitnall 1988; Whitnall 1989; de Goeij *et al.* 1992; Bartanusz *et al.* 1993; Paulmyer-Lacroix *et al.* 1994).

Vasopressin alone is a less effective stimulator of ACTH secretion, but is the most potent among the co-secretagogues that act synergistically with CRH to induce ACTH secretion (Gillies *et al.* 1982; Bruhn *et al.* 1984).

During pregnancy, the HPA axis responses to physical and emotional stress are attenuated (Neumann *et al.* 1998), which could be partly due the hypothalamic secretagogue inputs. There is some evidence of lower basal vasopressin mRNA expression in the parvocellular PVN of pregnant rats, suggesting reduced vasopressin availability for driving the secretion of ACTH (Johnstone *et al.* 2000). However, the roles of vasopressin on ACTH secretion *in vivo* during pregnancy are not clear. Exogenous vasopressin administration and blocking the actions of vasopressin *in vivo* with a potent V1a/b receptor antagonist, dP(Tyr(Me)$_2$,Arg-NH$_2^9$)AVP (Manning *et al.*

1992), are useful pharmacological tools for studies on vasopressin effects on the V1b receptor-mediated release of ACTH from corticotrophs.

The sensitivity of corticotrophs during pregnancy may be changed as suggested from the receptor autoradiography studies: vasopressin receptor binding is decreased in the anterior pituitary in pregnant rats compared with virgins (Toufexis *et al.* 1999), but there is no direct evidence on whether and how the sensitivity is changed during pregnancy.

5.2 Aims

The studies in this chapter were to elucidate the role of vasopressin by giving exogenous vasopressin and by blocking vasopressin effect with V1a/b receptor antagonist, and to examine the sensitivity of corticotrophs by injecting both CRH and vasopressin together in the attenuated ACTH response to stress during pregnancy.

5.3 Materials and methods

ACTH stimulation by exogenous vasopressin in vivo

To study vasopressin action on ACTH secretion, individually caged pregnant and virgin animals had a silastic jugular cannula implanted as described in Chapter 2.1.2.1. The experiment was carried out four to five days following surgery to allow the animals to recover. On the day of experiment (day 21 of pregnancy), the jugular cannula was flushed and connected to a syringe filled with sterile heparinised saline (at 0800 h). Two basal blood samples (0.4 ml) were taken 1 and 1.5 h later. Immediately after the second basal sample, each rat received either vasopressin (1.7 μg/kg; after D.J.Toufexis (Toufexis *et al.* 1999)) or saline vehicle (500 μl/kg) intravenously, and further blood samples were taken after 5, 15, 30 and 60 min. The aim was to achieve circulating levels of vasopressin which were identical to to those in the hypophyseal vessels. Each blood sample was collected into a tube containing 15

μl 5% EDTA per 100 μl blood. Blood was replaced with 0.9% saline. Blood samples were cooled on ice, centrifuged at 12,000 g, and plasma separated and stored at -70°C until radioimmunoassay for ACTH.

Vasopressin receptor antagonist and stress-stimulated ACTH secretion

Virgin and pregnant rats were implanted with a jugular cannula, connected as in the vasopressin study. After two basal blood samples (0.4 ml) were taken at 08.30- 09.00 h 60 min and 90 min after connection, the vasopressin receptor antagonist [dP(Tyr(Me)^2,Arg-NH_2^9)AVP, (10 μg/kg, i.v. MW= 1210.5, a mixed V1a and V1b receptor antagonist, with essentially no V2 activity, kindly donated by Prof. M. Manning) or vehicle (500 μl/kg) was given.

The effective dose of this antagonist on V1a receptors *in vivo*, to reduce the effectiveness of vasopressin on blood pressure by 50%, is 0.21±0.02 nmol/kg (Manning *et al.* 1992). *In vitro*, tested in a radioligand competition study, this compound's affinity to V1a receptors (in rat liver) is 26.8 times greater than to V1b receptors (in porcine pituitary cells) (Arsenijevic *et al.* 1994). So the expected effective concentration for blocking vasopressin binding to V1b receptors in the anterior pituitary was calculated to be 6.8 μg/kg (26.8 x 0.21 nmol/kg, with MW: 1210.5). In a previous *in vivo* study, 10 μg/kg of a related compound (d(CH_2)$_5$, Tyr(Me)AVP) was found to prevent stimulation by CRH (after dexamethasone suppression) of ACTH secretion in aged, but not young rats (Hatzinger et al, 2000). It reduced blood pressure and increased heart rate similarly in aged and young rats, indicating that its actions on ACTH secretion were via V1b receptors in the anterior pituitary. The compound used in the present study was selected for its greater affinity at V1b receptors than d(CH_2)$_5$, Tyr(Me)AVP, and its relatively greater effectiveness on V1b receptors (Arsenijevic *et al.,* 1994). The peak concentration of the antagonist in extracellular fluid (volume=67ml/ kg (Dyckes DF *et al*, 1974)) at the dose used in this study is calculated to be 120 nmol/l. This is less than the concentration that was found to be effective *in vitro* in blocking the augmentation by vasopressin of CRH-stimulated ACTH secretion (between 100 [no effect] and 10000 nmol/l, Chapter 6).

Immediately after the third basal sample was taken 15 min later, all the rats were forced to swim for 90 s in deep water (19°C), and further blood samples were taken

after 5, 15 and 60 min. To test the effectiveness of the antagonist, immediately after the 60 min sample, another injection of vasopressin receptor antagonist (10 μg/kg) or saline vehicle (500 μl/kg) was given; 15 min later, a blood sample was taken, then vasopressin (1.7 μg/kg) was given to all rats and 10 min after the vasopressin injection, a blood sample was taken. Each blood sample was collected into a tube containing 15 μl 5% EDTA per 100 μl blood. Blood was replaced with 0.9% saline. Blood samples were cooled on ice, centrifuged at 12,000 g for 5 min and plasma separated and stored at -70°C until assayed.

The combination of CRH and vasopressin on ACTH secretion in vivo

To study the effect of a combination of exogenous CRH and vasopressin on ACTH secretion, virgin and pregnant rats were implanted with a jugular cannula, connected as in the vasopressin alone study. Blood samples were taken, stored and assayed as in the exogenous vasopressin administration study but each rat received a combined intravenous injection of CRH (200 ng/kg) and vasopressin (1.7 μg/kg) instead.

At the end of all of the experiments, the animals were killed by an intravenous overdose of anaesthetic (Sagatal, 36 mg in 600 μl per rat) and pregnancy status was checked. ACTH concentrations were assayed with the ACTH kits from ICN (for details see Chapter 2.2.2.5).

Statistics

Data were analysed with two-way ANOVA for repeated measurements followed by post-hoc Student-Newman-Keuls tests, except data from the ACTH increment at 5 min post-swim stress after V1a/b receptor antagonist administration, which were analysed with one-way ANOVA followed by post-hoc Student-Newman-Keuls test.

5.4 Results

Pituitary ACTH response to exogenous vasopressin administration

There were no differences in basal concentrations of ACTH between virgin and pregnant rats. The plasma concentration of ACTH was increased significantly following vasopressin injection in both virgin and pregnant rats (Figure.5.1). Both virgin and pregnant rats exhibited significant increases in plasma ACTH concentrations at 5 min and 15 min after vasopressin injection ($p<0.0001$, two-way ANOVA followed for repeated measurements by post-hoc Student-Newman-Keuls tests). Peak secretion was seen at the 5-min time point in both virgin and pregnant rats. In both virgin and pregnant rats, there was no ACTH response to vehicle injection (plasma ACTH: basal: virgins=56.2±12.3 pg/ml n=6; pregnant=52.3±11.0 pg/ml n=7; 5 min after vehicle: virgins=64.0±5.0 pg/ml, n=7; pregnant=48.5±12.9 pg/ml, n=9, $p>0.05$). Vasopressin stimulated a large ACTH release that was significantly different at 5 min ($p<0.01$), and 15 min ($p<0.05$) in virgin compared with pregnant rats (ACTH at 5 min: virgins=1414.5±260.8 pg/ml, n=7, pregnant=791.4±76.5 pg/ml, n=9, $p<0.05$). Vasopressin-administrated rats lay prostrate for several minutes after injection: perhaps a consequence of vasopressor actions.

Effect of vasopressin 1 a/b receptor antagonist on ACTH response to swimming stress

The results are shown in Figure 5.2. Two-way ANOVA for repeated measurements followed by post-hoc Student-Newman-Keuls test showed a significant interaction between time and group for plasma ACTH concentration (Figure 5.2, $p<0.0001$). There was no significant difference in basal ACTH levels between virgin and pregnant rats ($p>0.05$). The administration of the V1a/b receptor antagonist did not change plasma ACTH levels in any group 15 min after injection. Swim stress increased plasma ACTH concentrations at 5 and 15 min in all groups ($p<0.05$). The peak ACTH response occurred at 5 min after swimming in all groups ($p<0.05$). Five min after the end of the 90 s swim stress, ACTH concentrations were significantly lower in the V1a/b receptor antagonist- treated virgin rats compared with the vehicle-treated virgin rats ($p<0.05$). However, the V1a/b receptor antagonist treatment did not reduce plasma ACTH concentration in pregnant rats at 5- min time point ($p>0.05$).

Figure 5.1 ACTH response to vasopressin *in vivo* in virgin and pregnant rats

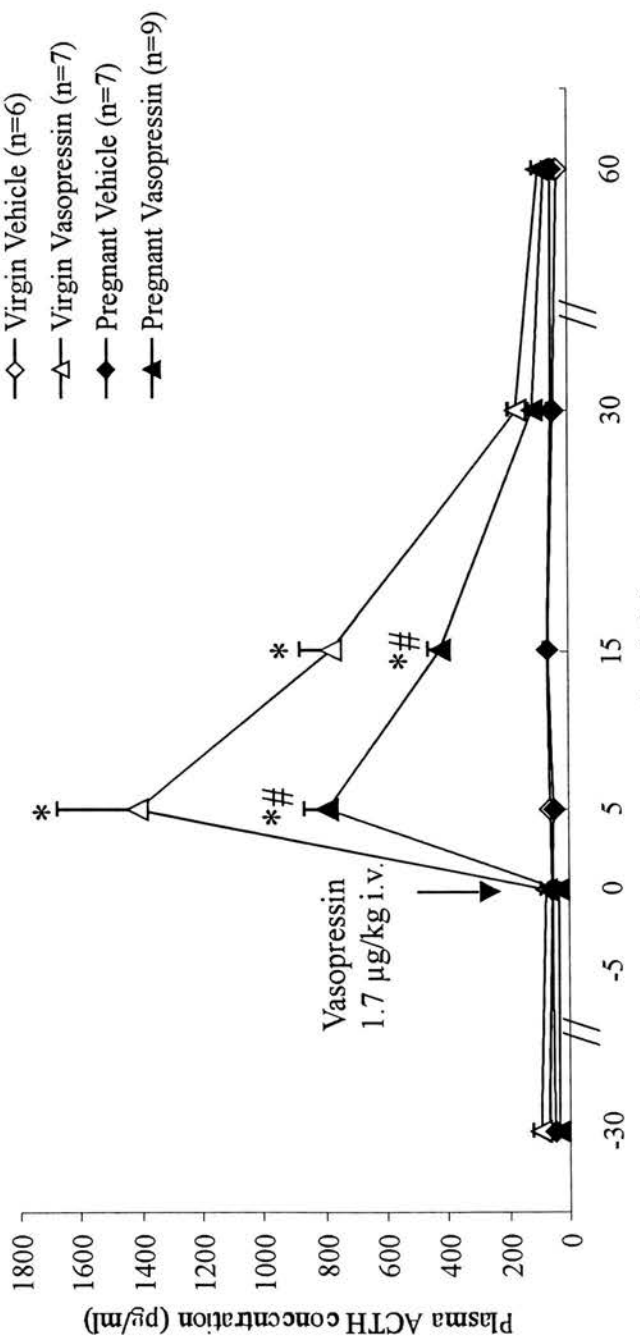


Figure 5.1. The effect of vasopressin on plasma ACTH concentrations in pregnant (day 21) and virgin rats. Basal blood samples were collected from a jugular vein 30 min and immediately before vasopressin bolus injection (1.7 µg/kg). Then further blood samples were taken 5 min, 15 min, 30 min and 60 min after vasopressin administration. Values are means ±SEM. Statistical analysis: significant effect of vasopressin ($p<0.05$, two-way ANOVA for repeated measurements followed by Student-Newman-Keuls tests) and of pregnancy (day 21) ($p<0.05$). *$p<0.05$ *vs* basal, #$p<0.05$ *vs* virgin, two-way ANOVA for repeated measurements followed by Student-Newman-Keuls tests.

Compared to the respective vehicle-treated groups, 15 min after stress there were no differences between the antagonist and respective vehicle-treated groups, although there were still significant differences between vehicle- treated pregnant and virgin rats at this time point. By 60 min post stress, plasma ACTH concentrations had returned to near basal and were not significantly different among groups, although in the pregnant-vehicle group, ACTH concentration was not less than at 15 min.

Increases in plasma ACTH concentration at 5 min after swim stress are expressed relative to the pre-swimming basal value in Figure 5.3. Swimming increased plasma ACTH concentration more in the vehicle-injected virgin that in the vehicle-injected pregnant rats. There was significant inhibition of the ACTH response to swimming stress by the V1a/b receptor antagonist in virgin rats ($p<0.05$), but not in the pregnant group.

To test the effectiveness of the vasopressin antagonist on vasopressin actions on ACTH secretion, the same dose of the vasopressin antagonist or vehicle was given intravenously to the treatment group or vehicle group respectively at 60 min after stress. 15 min later, a dose of vasopressin (1.7 μg/kg) was given to all groups (Figure 5.4). Vasopressin induced a significant increase in ACTH in both virgin and pregnant vehicle-treated groups. Consistent with the above study of the effects of exogenous vasopressin, vasopressin at this dose caused a much greater ACTH response in the vehicle-treated virgin rats compared with the vehicle-treated pregnant rats. The V1a/b receptor antagonist, (dP(Tyr(Me)^2,Arg-NH_2^9)AVP, largely prevented (by 89.5% and 91.3% in virgin and pregnant rats respectively) the ACTH response to vasopressin in both virgin and pregnant groups.

It was observed that rats injected with vasopressin rapidly adopted a prostrate posture, resuming a normal posture after several minutes. This effect of vasopressin was completely prevented by prior injection of the V1a/b receptor antagonist.

Figure 5.2 Vasopressin receptor antagonist on stress-induced ACTH secretion *in vivo* in virgin and pregnant rats

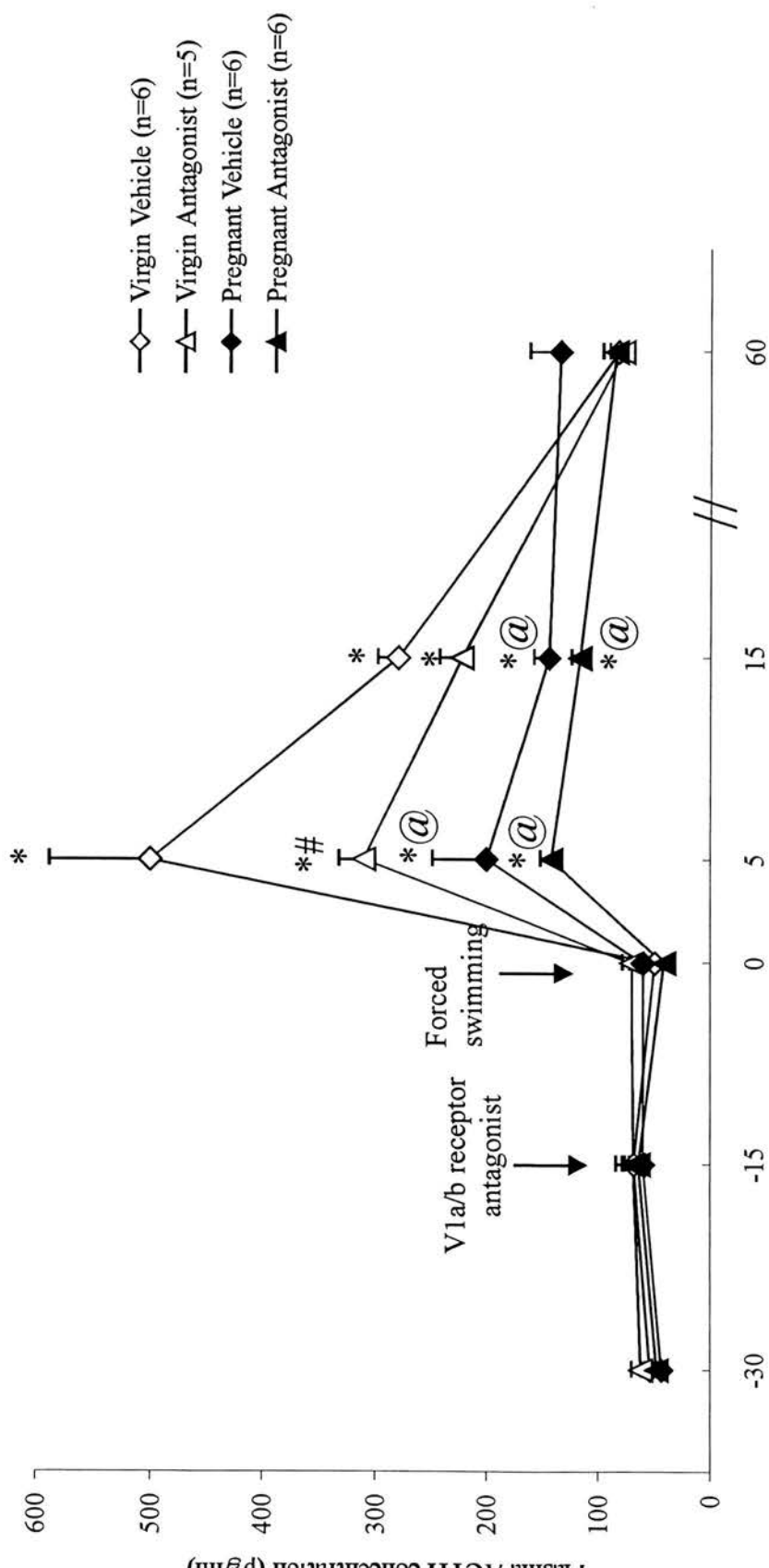


Figure 5.2 Effect of vasopressin receptor antagonist on ACTH secretion. Data are means ±SEM. 15 min after vasopressin antagonist (10 µg/kg, iv) or vehicle injection and the collection of three basal blood samples, all rats were forced to swim for 90 s. Statistical analysis: significant effects of swimming at 5 and 15 min ($p< 0.0001$, two-way ANOVA for repeated measurements), of pregnancy (day 21) at 5 and 15 min ($p<0.05$), and of vasopressin receptor antagonist on the stress responses in virgins at 5 min ($p<0.05$). *$p<0.05$ *vs* basal, #$p<0.05$ *vs* respective vehicle, @$p<0.05$ *vs* virgin vehicle, two-way ANOVA for repeated measurements followed by Student-Newman-Keuls tests.

Figure 5.3 Increases in ACTH concentrations at 5 min post-swim stress in virgin and pregnant rats

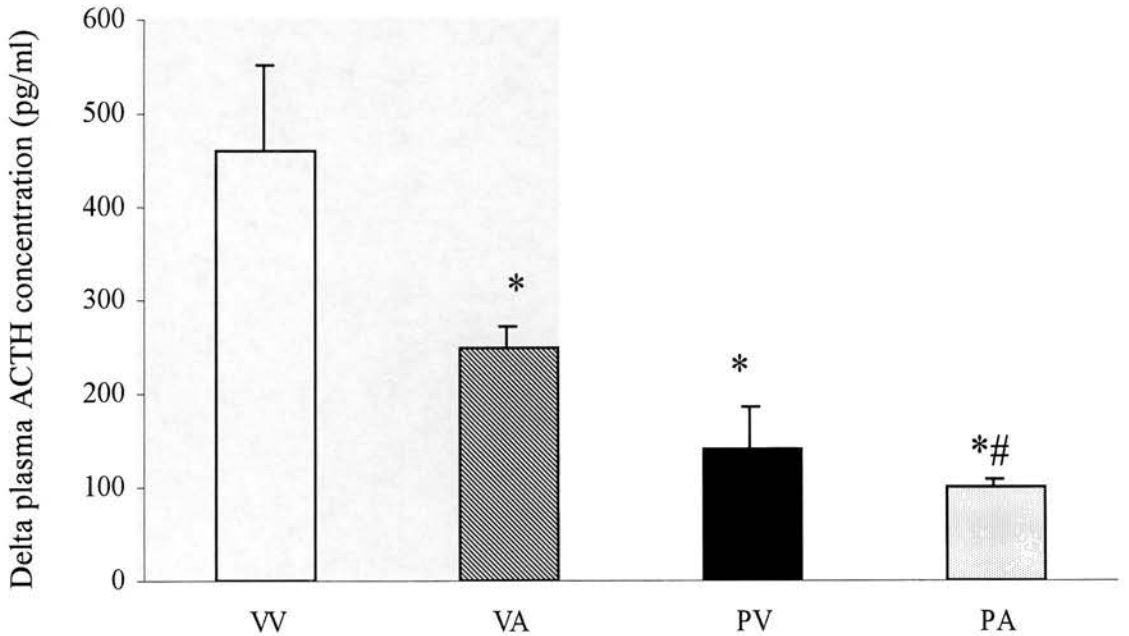


Figure 5.3 The histogram shows differences between basal and 5 min post-swim plasma ACTH concentrations (see Figure 5.2). The V1a/b receptor antagonist reduced the ACTH response to swim stress in virgin (*$p<0.05$ *vs* virgin vehicle group, #$p<0.05$ *vs* virgin antagonist group, one-way ANOVA), but not in day 21 pregnant rats. VV=virgin vehicle (n=6), VA=virgin vasopressin receptor antagonist (n=5), PV=pregnant vehicle (n=6), PA=pregnant vasopressin receptor antagonist (n=6).

Figure 5.4 Effect of V1a/b receptor antagonist on exogenous vasopressin action

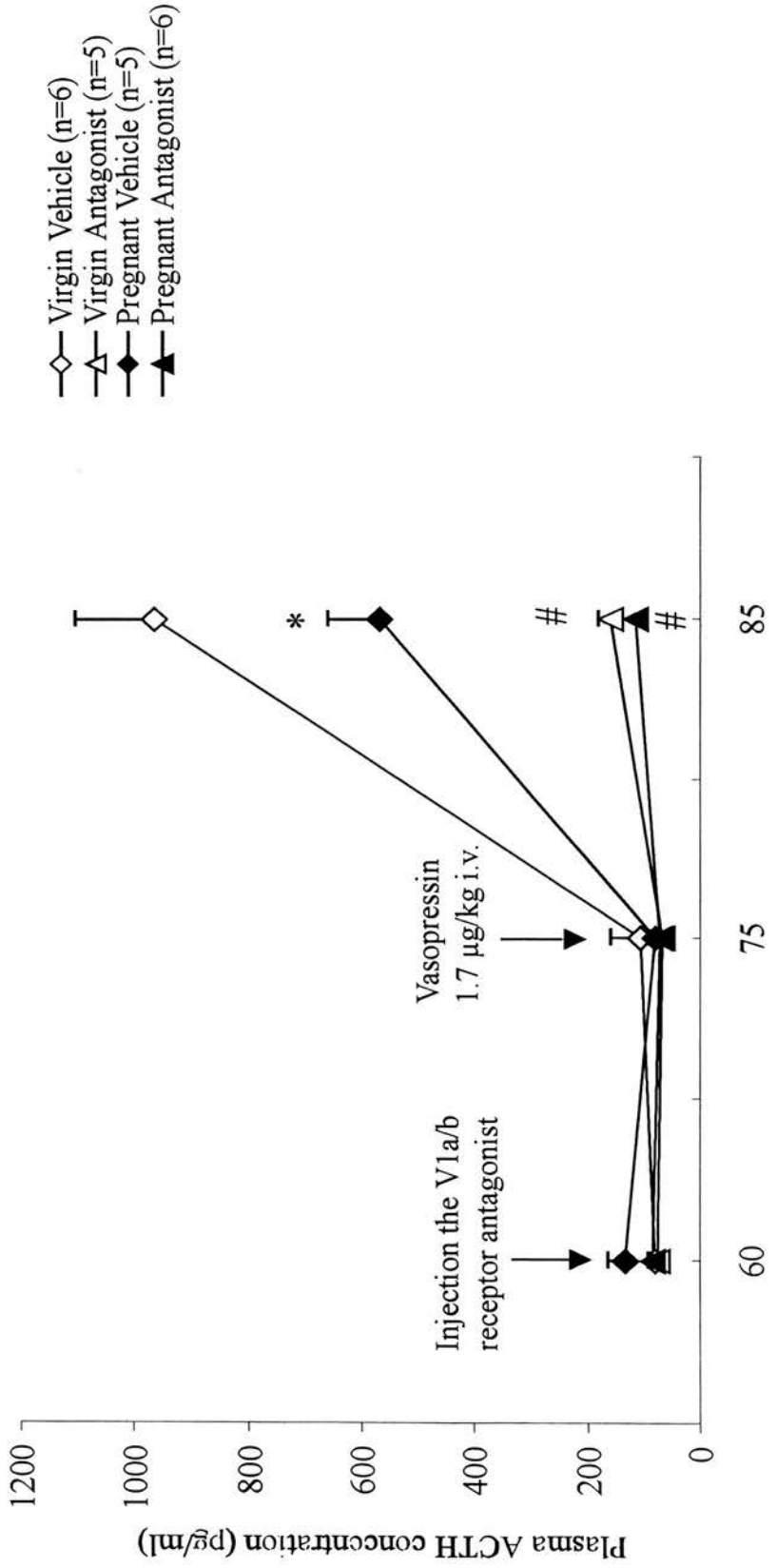


Figure 5.4 Effect of the V1a/b receptor antagonist on exogenous vasopressin action. Same experiment as Figure 5.2: the antagonist treated rats were given a second injection of V1a/b receptor antagonist (10 µg/kg) at 60 min. Immediately after the 60 min blood sample was taken, all rats were given vasopressin intravenously. A further blood sample was taken 10 min later. ACTH concentrations were significantly greater in the vehicle groups than those in the V1a/b receptor antagonist treated groups (#$p<0.05$ *vs* respective vehicle groups, two-way ANOVA for repeated measurements). In the vehicle groups, ACTH concentrations in response to the exogenous vasopressin in the virgin rats were greater than those in the day 21 pregnant rats (*$p<0.05$ *v s* virgin vehicle group, , two-way ANOVA for repeated measurements).

Combined CRH and vasopressin injection on ACTH secretion in vivo

A combination of CRH (200 ng/kg, i.v., same dose as the CRH study in Chapter 4) and vasopressin (1.7 μg/kg, i.v., same dose as above vasopressin study) significantly increased plasma ACTH concentrations at 5 min and 15 min after injection (Figure 5.5, $p<0.01$). The peak ACTH secretion was observed at the 5-min time point for both groups. Plasma ACTH concentrations in the virgin and pregnant groups were not different from each other at any time point. ($p>0.05$, two-way ANOVA for repeated measurements).

5.5 Discussion

Reduced effect of exogenous vasopressin on ACTH secretion in vivo in pregnancy

In this study, we applied exogenous vasopressin (1.7 μg/kg) at a dose aimed to mimic hypothalamic secretion of vasopressin in stress, which elicits a high ACTH response in male and female rats (Toufexis *et al.* 1999). Like the response to CRH, in response to exogenous vasopressin, the magnitude of the plasma ACTH response was also attenuated in late pregnancy compared to virgin rats. This phenomenon suggests a decrease in pituitary sensitivity to vasopressin, and/or a decrease in CRH secretion in the portal blood occurs under basal conditions during pregnancy. The robust increase in ACTH release after exogenous vasopressin injection greatly exceeded peak amounts normally seen in virgin and pregnant rats during stress or after exogenous administration of CRH. Part of the effect of vasopressin on ACTH secretion may have been indirect, via the stimulation of hypothalamic CRH neurones (Laszlo *et al.* 2001), consequent on vasoconstrictor action of vasopressin through other effects, such as discomfort. The indirect action of vasopressin on ACTH secretion may be significant when vasopressin is administrated *in vivo*, because it is known to have marked cardiovascular effects through V1a receptors even in low concentrations (Mormede *et al.* 1985) and V1a receptor antagonist is effective in inhibiting the release of ACTH (Rivier & Vale 1983).

Furthermore cardiovascular effects of vasopressin are reduced in pregnancy (Paller 1984), so differences between pregnant and virgin rats could be partly a consequence of different central processing of inputs from the vascular effects of vasopressin. We

Figure 5.5 Effect of CRH and vasopressin together on ACTH secretion during pregnancy *in vivo*

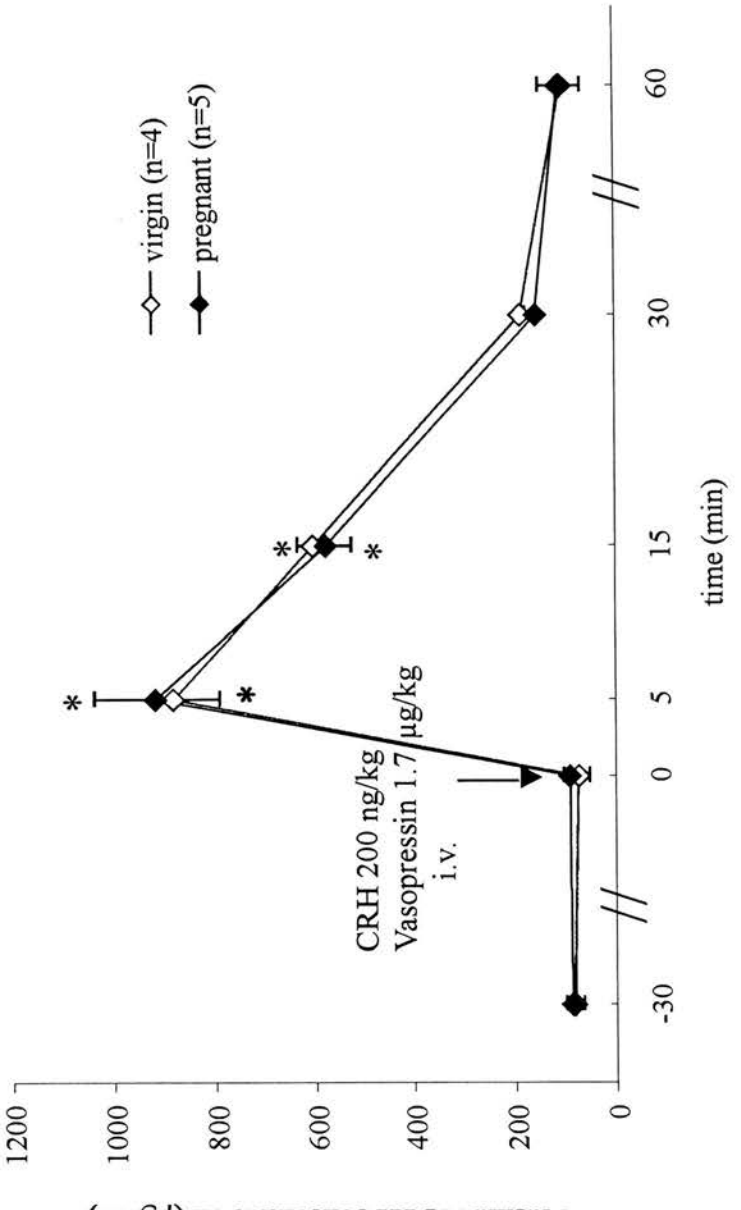


Figure 5.5 The effect of CRH and vasopressin together on ACTH secretion *in vivo*. CRH (200 ng/kg) and vasopressin (1.7 µg/kg) were injected i.v. immediately after collection of two basal blood samples (at –30 and 0 min). Data are means ± SEM. Statistical analysis: CRH and vasopressin together increased ACTH levels in both virgin and pregnant rats at 5, 15 min after injection (*$p<0.01$ *vs* the basal values, two-way ANOVA for repeated measurements); there were no differences in the effects of CRH + vasopressin ($p>0.05$) between virgin and day 21 pregnant rats.

have not studied this further. The V1a receptor mediates the vasoconstrictor and hepatic glycogenolytic action of vasopressin (Fishman *et al.* 1987; Estrada *et al.* 1991), but differences between pregnant and virgin rats were greatest at just 5 min.

Similar to the results from study of CRH effects on ACTH secretion, the results in this study showed that the ACTH response to vasopressin in the pregnant rats was decreased compared with the virgins. This suggests that a possible reduced sensitivity to exogenous ACTH secretagogues exists in pregnant rat corticotrophs. Vasopressin potentiates CRH effects on ACTH secretion (Gillies *et al.* 1982; Bruhn *et al.* 1984; Norman & Challis 1987). So the reduced ACTH secretion in response to exogenous vasopressin in pregnancy would have been the result of the interaction with endogenous CRH. The results in Chapter 4 show that the CRH content in the median eminence was less in the late pregnant rats, and this may indicate that CRH production and the release of CRH into the portal blood, under basal conditions, is less as well. The reduced ACTH secretory response may be the result of interaction of the reduced endogenous CRH release and the same concentration of exogenous vasopressin.

The endogenous CRH and vasopressin concentrations in the portal blood in pregnant rats are not known. Vasopressin stores in the external zone of the median eminence in the parvocellular terminals is considered to be responsible for pituitary ACTH release, whereas vasopressin produced in magnocellular neurones of the PVN and SON is transported via nerve fibres in the internal zone of the median eminence to the posterior pituitary lobe and secreted into the peripheral circulation in response to osmotic and related stimuli (Antoni 1986; Holmes *et al.* 1986; Morris & Pow 1988; Antoni *et al.* 1990). As vasopressin in the median eminence is from both parvocellular and magnocelluar origin, it is difficult to assess any change in stored vasopressin of parvocellular origin by measuring vasopressin content here. A change in vasopressin content in the external zone of the median eminence could be investigated by immunohistochemistry methods. However, a reduced secretion into portal blood of CRH and vasopressin is suggested by reduced vasopressin and CRH mRNA expression in PVN neurones in pregnancy (Johnstone *et al.* 2000).

Effect of the V1a/b receptor antagonist on ACTH secretion in vivo in pregnancy

This V1a/b receptor antagonist should not enter the brain from the blood as it is a peptide, so it exerts its anti-ACTH secretory function through V1b receptors on corticotrophs directly and through V1a receptor on blood vessels indirectly (Manning *et al.* 1992). Actions of the V1a/b antagonist on corticotrophs were shown *in vitro* (see Chapter 6). In the present study, as the V1a/b antagonist decreased the ACTH response to swim stress in virgin but not in pregnant rats (Figure 5.3) and as exogenous vasopressin induced less ACTH secretion in pregnancy (Figure 5.1), this implies that vasopressin is less effective in pregnancy, or that less is released in response to swim stress.

The V1a/b receptor antagonist was very effective in blocking the ACTH response to exogenous vasopressin both in virgin and in pregnant rats (Figure 5.4). The V1a/b receptor antagonist reduced the ACTH stress response in virgin, but not in pregnant rats, so it seems reasonable to conclude that vasopressin secretion contributes less to the stimulation of ACTH secretion in response to stress in pregnant than in virgin rats.

Interestingly, the vasopressin receptor antagonist decreased the stress response at 5, and not at 15 min in virgin rats. It is in contrast to the CRH antagonist, which decreased this response at both 5 min and 15 min both in virgin and pregnant rats. This suggests that vasopressin is more important in the rapid ACTH response to the stress.

The rats developed a prostrate posture soon after injection of vasopressin alone intravenously. This effect was not seen after administration of the V1a/b receptor antagonist prior to vasopressin injection. This may have been a consequence of blocking pressor effects of vasopressin through V1a receptors (Ervin *et al.* 1993). Without a selective V1a or V1b receptor antagonist, we have not investigated this issue further. However, the antagonist was as effective in pregnant and virgin rats in blocking this response as well as blocking ACTH secretion, yet ineffective in reducing ACTH secretion in response to stress in pregnant rats in contrast with virgins, and that V1b action of the antagonist were shown *in vitro* (Chapter 6).

In addition to decreased vasopressin secretion in pregnancy, a small difference in effective V1b receptor number on corticotroph cells (Toufexis *et al.* 1999) may be crucially important for the different ACTH response to stress in pregnancy because of the synergistic effect of vasopressin on CRH (Gillies *et al.* 1982). The V1b receptor content in the anterior pituitary of pregnant rats is reduced compared with virgin rats (Toufexis *et al.* 1999), as is V1b receptor mRNA expression (Chapter 3). This suggests the sensitivity of corticotroph cells to vasopressin might decrease during pregnancy.

No difference in exogenous CRH plus vasopressin on ACTH secretion in vivo between virgin and pregnancy

To test the sensitivity of corticotrophs *in vivo*, we gave CRH and vasopressin in combination at the same doses as in the previous two studies (Chapter 4 and this chapter). The lack of a difference between virgin and pregnant rats in the ACTH response to this combined administration of CRH and vasopressin indicates that the reduced response to either alone in pregnancy is due to reduced basal CRH or vasopressin secretion, or both, and that the sensitivity of the corticotrophs to the secretagogues might not be decreased during pregnancy. However, not ruled out is the possibility of a change in CRH and vasopressin interaction at the pituitary during pregnancy (see Chapter 6, *in vitro* studies).

Nonetheless, in summary, the similar ACTH response to combined injection of CRH and vasopressin in virgin and pregnant rats sugests that: 1) the secretory capacity of the corticotrophs *in vivo* in pregnancy is not diminished; 2) it is likely that the reduced effect in pregnancy of CRH or vasopressin alone on ACTH secretion is a consequence of reduced basal endogenous secretion of vasopressin or CRH respectively; 3) the *in vitro* studies (Chapter 6) of interactions between CRH and vasopressin in pregnancy can be extended to the *in vivo* situation; 4) as there are changes in expression of mRNAs related to signalling and secretion in corticotrophs in pregnancy (Chapter 3), these adaptations maintain secretagogue responsiveness, even though endogenous CRH and vasopressin secretion are reduced; 5) during stress it seems that reduced ACTH secretion is primarily a consequence of reduced vasopressin secretion by the parvocellular PVN neurones.

Chapter 6

Cellular signalling in corticotrophs *in vitro*

6.1 Introduction

CRH and vasopressin are the major regulators of the secretion of ACTH from the corticotrophs in the anterior pituitary (Antoni 1986). CRH is the most potent stimulator of POMC transcription and ACTH biosynthesis and secretion from the anterior pituitary (Vale *et al.* 1981; Antoni 1986) via the stimulation of CRHR1 (Chen *et al.* 1993). In the corticotrophs, CRHR1 is positively coupled to the cAMP signalling cascade (Labrie *et al.* 1982), leading to a transient increase in intracellular cAMP concentrations (Litvin *et al.* 1984; Battaglia *et al.* 1987). This increase in intracellular cAMP accumulation stimulates ACTH biosynthesis and secretion (Antoni 1986; King & Baertschi 1990).

Vasopressin from parvocellular PVN neurones is responsible for regulating ACTH secretion (Aguilera 1994) and this action of vasopressin on the corticotrophs is mediated by V1b receptors. Vasopressin greatly potentiates the ACTH response to CRH *in vivo* and *in vitro* in rats (Gillies *et al.* 1982; Fischman & Moldow 1984). This potentiating action of vasopressin involves increase in cAMP formation by stimulation of adenylate cyclase (Abou-Samra *et al.* 1987) and a decrease in cAMP degradation by inhibiting phosphodiesterase activity (Giguere & Labrie 1982) in rat anterior pituitary cells. V1 receptor antagonists are effective in inhibiting the release of ACTH (Rivier *et al.* 1984; Buckingham 1987; Watanabe *et al.* 1989). A V1a/b receptor antagonist, dP(Tyr(Me)^2,Arg-NH_2^{9}]AVP (Manning *et al.* 1992) is useful in studies on the role of vasopressin in the V1b receptor- mediated release of ACTH from corticotrophs (see Chapter 5).

During pregnancy, the ACTH response to stress and exogenous CRH is attenuated (Neumann *et al.* 1998). It is reported that cAMP accumulation stimulated by CRH in

segments of anterior pituitaries from the pregnant rats is reduced compared with those from virgin rats (Neumann *et al.* 1998) and that CRH receptor binding in the anterior pituitaries of the pregnant rats is also decreased, which suggests reduced CRH signalling in the corticotrophs of the pregnant rats. However, whether CRH cellular signalling transduction via cAMP results altered ACTH release or whether CRH/vasopressin interaction is changed is not known.

6.2 Aims:

In vitro studies are useful for understanding CRH and vasopressin signalling and their interaction on ACTH secretion in corticotrophs by excluding the interference of other pathways. This study was to investigate the sensitivity of the corticotrophs *in vitro* and to find any cellular signalling changes in pregnancy. We measured ACTH secretion from acutely isolated anterior pituitary cells incubated with CRH, vasopressin, CRH and vasopressin together, and with a cell membrane permeable cAMP analogue (8-CPT-cAMP).

6.3 Materials and methods

Details of measurement of ACTH release from acutely isolated anterior pituitary cells are in Chapter 2.2.2.1. Briefly here, the anterior pituitary glands were removed from decapitated virgin and pregnant (day 10 and day 21, around 0900 h), and acutely dispersed, and incubated with vehicle (DMEM-BSA), ACTH secretagogues, or 8-CPT-cAMP (a cell membrane permeable cAMP analogue) in the study of vasopressin and CRH interaction *in vitro.* In the case of measurement of the effects of the V1a/b receptor antagonist, on ACTH secretion by corticotrophs from virgin rats, medium containing [dP(Tyr(Me)^2,Arg-NH_2^{9})AVP was added to tubes containing cells, which were left on ice; 5 min later the secretagogues were then added, and the cells incubated for 1 h at 37°C in a shaking water bath. To measure total ACTH content of cells, 200 µl aliquots (100,000 cells) were frozen directly at –70°C without any incubation or addition of secretagogues. Samples were stored at –70°C until radioimmunoassay.

ACTH concentrations were measured using a double antibody precipitation radioimmunoassay (Dayanithi & Antoni 1989) after 5 times dilution in RIA buffer (details in Chapter 2.2.2.2). The concentration of ACTH was calculated as in Chapter 2.2.2.3.

Statistics

The secretion and ACTH concentration experiments were repeated 4-7 times, to explore different features of changes in pregnancy. Within an experiment, the data were analysed with two-way ANOVA with post-hoc Student-Newman-Keuls Method. The summary data were analysed with Kruskal-Wallis One-way ANOVA on Ranks and followed by Mann-Whitney Rank Sum Test.

Data from corticotroph ACTH content measurement were analysed with t-test.

The data from the V1a/b receptor antagonist measurement in virgin rats were analysed with one-way ANOVA with post-hoc Student-Newman-Keuls Method.

6.4 Results

The sensitivity of anterior pituitary cells to CRH, vasopressin, the combination of CRH and vasopressin, and cAMP in virgin and day 21 pregnant rats

Dispersed anterior pituitary cells were incubated in vehicle (DMEM-BSA), CRH alone, vasopressin alone or CRH plus vasopressin. ACTH release was calculated per 100,000 cells. For CRH alone, for both virgin and pregnant rats, ACTH secretion was dose-dependently related to CRH concentration (Figure 6.1). Vasopressin alone had no effect on ACTH secretion at the doses used. Vasopressin (2 nM) potentiated the stimulatory effect of CRH (0.1 nM and 1 nM) on ACTH secretion ($p<0.05$), and this augmentation effect was greater in pregnant rats ($p<0.05$, two-way ANOVA). 8-CPT-cAMP treatment also caused ACTH release. The effects of 8-CPT-cAMP were greater on cells from pregnant rats ($p<0.05$, two-way ANOVA). To correct for differences ($p>0.05$) in basal release between cells from virgin and pregnant rats, ratios were calculated by comparing all values with the basal ones. The pattern of the data presented by ratio (Figure 6.2) showed greater effects of CRH alone on cells from

Figure 6.1 A representative figure of ACTH secretion from isolated virgin and pregnant rat anterior pituitary cells

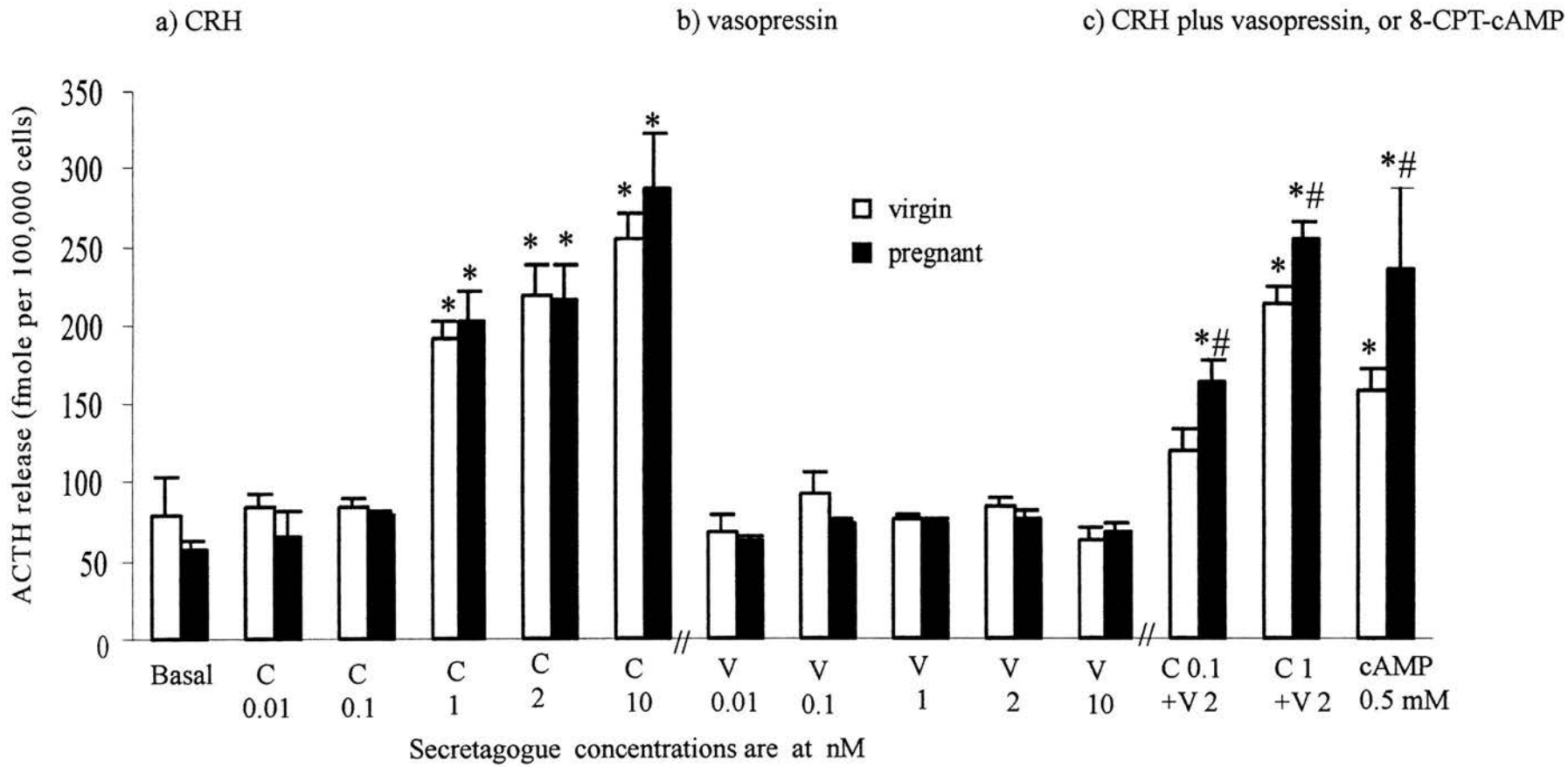


Figure 6.1 A representative figure of the effect of CRH, vasopressin, the combination of CRH and vasopressin, and 8-CPT-cAMP on ACTH secretion from acutely dispersed anterior pituitary cells. The data are the raw values expressed as mean ± SEM. In both virgin andday 21 pregnant groups: ACTH secretion showed a dose-dependent response to CRH; There is no apparent stimulatory effect of vasopressin alone; vasopressin potentiated the CRH effect on ACTH secretion.
There is no difference in response to CRH or vasopressin alone between the virgin and pregnant groups. However, in the pregnant group, the cells secreted more ACTH in response to the combination of CRH and vasopressin, and 8-cpt-cAMP (*$p<0.05$ *vs* respective basal; #$p<0.05$ *vs* respective virgin, two-way ANOVA; C=CRH, V=vasopressin; n=4 tubes for each treatment).

Figure 6.2 A representative figure of ratio of ACTH secretion from isolated virgin and pregnant rat pituitary cells

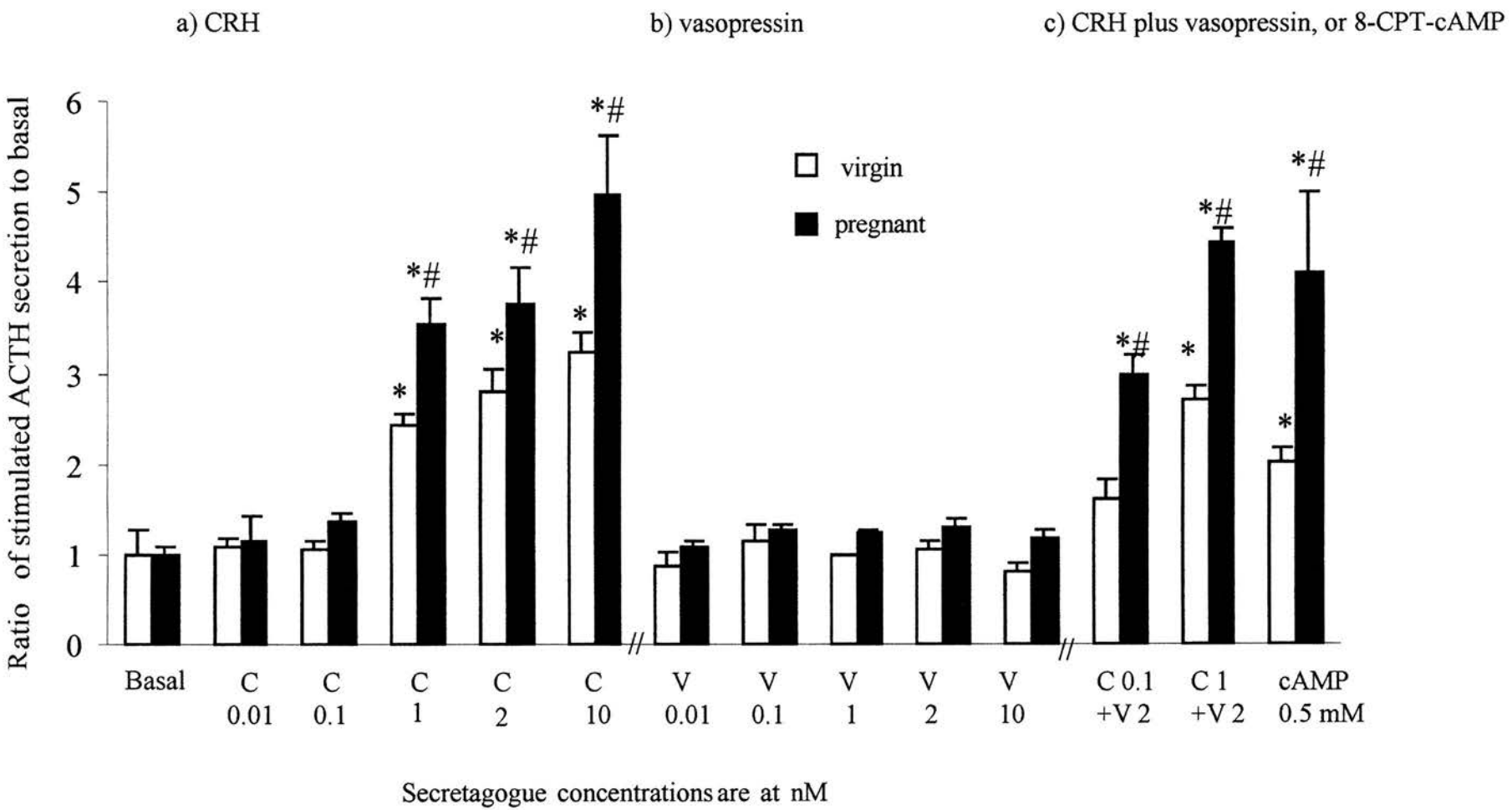


Figure 6.2 A representative figure of the effect of CRH, vasopressin, the combination of CRH and vasopressin, and cAMP on ACTH secretion from acutely dispersed anterior pituitary cells. The data are the ratios of individual value compared with basal, expressed as mean ± SEM (from data in Figure 6.1). In a similar pattern the data in Figure 6.1, in both virgin and day 21 pregnant groups: ACTH secretion showed a dose-dependent response to CRH; There is no apparent stimulatory effect of vasopressin; vasopressin potentiated the effect of CRH on ACTH secretion. However, in the pregnant group, the cells secreted more ACTH in response to CRH alone, the combination of CRH and vasopressin, and 8-CPT-cAMP (*$p<0.05$ *vs* respective basal; #$p<0.05$ *vs* respective virgin, two-way ANOVA; C= CRH, V= vasopressin; n=4 tubes for each treatment).

pregnant versus virgin rats, and greater vasopressin augmentation of CRH stimulation of ACTH secretion, and greater stimulation by 8-CPT-cAMP.

To enable combination of data from several experiments and thus compare the effects of secretagogues and cAMP on ACTH secretion by the virgin and pregnant pituitary cells across experiments, we calculated the ratio (R1) of total release of ACTH to basal release for each treatment. Then the ratio (R2) of pregnant to virgin was calculated by dividing the pregnant R1 by the virgin R1 for each treatment (to describe the difference in effects of treatment between pregnant and virgin rats; the virgin for R1 values were thus set to be 1). The mean of these values (R2) across experiments was then calculated. The summary data for day 21 and day 10 of pregnancy are shown (Figure 6.3 and Figure 6.5). In order to analyse the results, the data presented as ratios in Figure 6.3 and Figure 6.5 were compared with 1 (virgin), to test the null hypothesis that R2=1.

We also calculated the increment of each treatment compared with the basal (delta value). The difference between the pregnant delta value and the virgin delta value was then calculated for each treatment (Figure 6.4 and Figure 6.6, the virgin value was thus set to be 0). The data presented as the differences between pregnant delta values and virgin delta values were compared with 0 (virgin).

By the ratio calculation (Figure 6.3), the anterior pituitary cells from day 21 pregnant rats secreted more ACTH than the cells from virgin rats in response to the stimulation by 0.1 nM CRH, 1 nM CRH alone, and 0.1 nM CRH plus 2 nM vasopressin ($p<0.05$ *vs* control, Kruskal-Wallis One-way ANOVA on Ranks and followed by Mann-Whitney Rank Sum Test). Whereas by calculating the differences between the pregnant delta values and the virgin delta values (Figure 6.4), the results showed 0.1 nM CRH, 0.1 nM CRH plus 2 nM vasopressin, and 8-CPT-cAMP were more effective in stimulating ACTH secretion from day 21 pregnant rat anterior pituitary cells ($p<0.05$ *vs* control, Kruskal-Wallis One-way ANOVA on Ranks and followed by Mann-Whitney Rank Sum Test).

Figure 6.3 Combined data from all experiments: pregnant day 21 versus virgin ratio

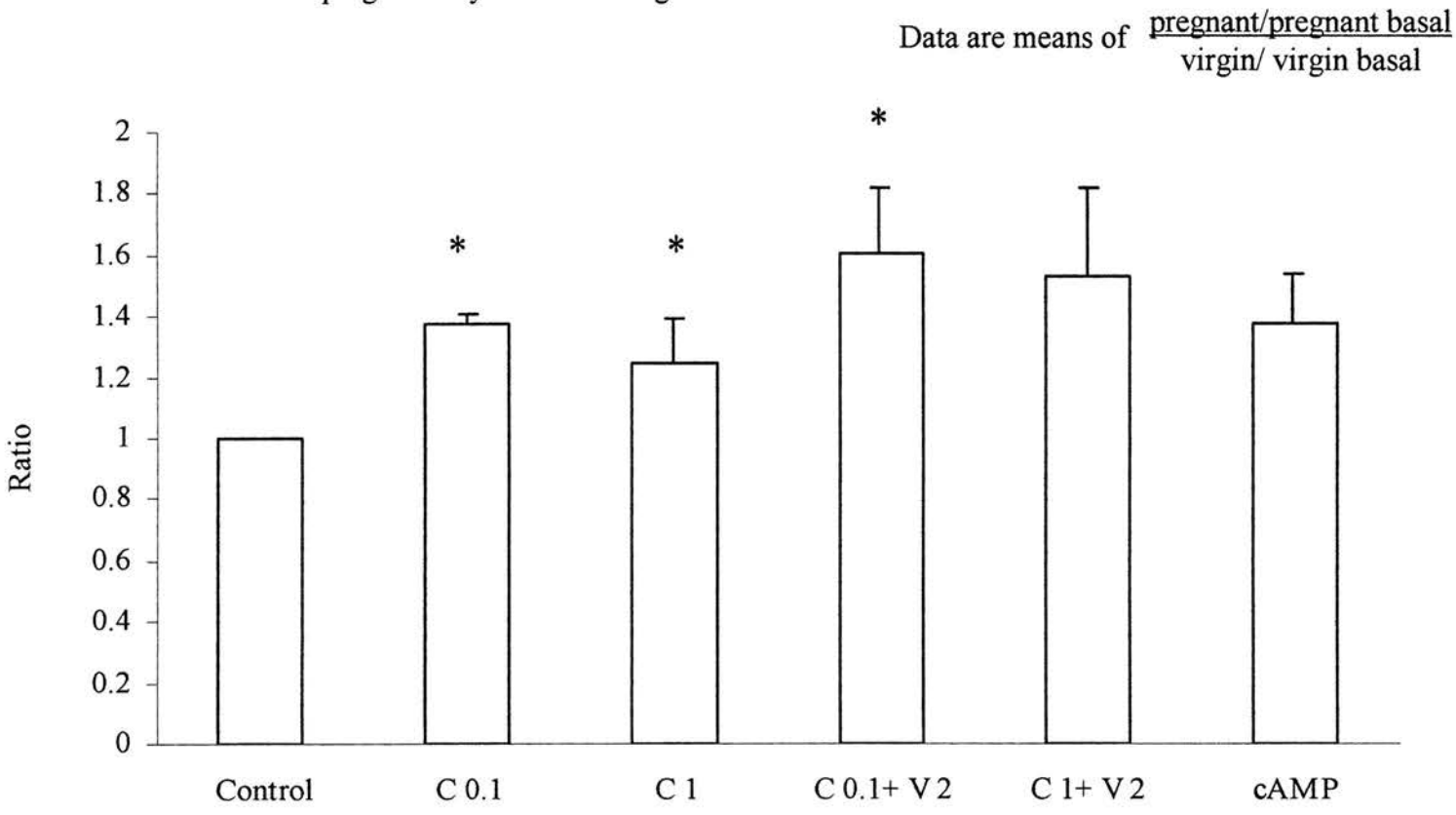


Figure 6.3 The effect of the secretagogues on ACTH secretion compared between the virgin and day 21 pregnant rats. The data in the histograms are means of ratio for pregnant versus virgin rats (based on respective ratio values). Compared with the virgin group (control, ratio= 1), CRH at dose of 0.1 nM and 1 nM, and the combination of 0.1 nM CRH and 2 nM vasopressin in the pregnant group stimulated more ACTH secretion (*$p<0.05$, Kruskal-Wallis One-way ANOVA on Ranks and followed by Mann-Whitney Rank Sum Test). 8-CPT-cAMP and the combination of 1 nM CRH and 2 nM vasopressin increased ACTH secretion more in the pregnant group, but this is not statistically significant. C 0.1= CRH 0.1 nM, n=4 experiments; C 1= CRH 1 nM, n=7 experiments; C 0.1+V 2= CRH 0.1 nM + vasopressin 2 nM, n=4 experiments; C 1+V 2= CRH 1 nM + vasopressin 2 nM, n=4 experiments; cAMP=8-CPT-cAMP, n=7 experiments.

Figure 6.4 Combined data from all experiments: pregnant day 21 versus virgin increments

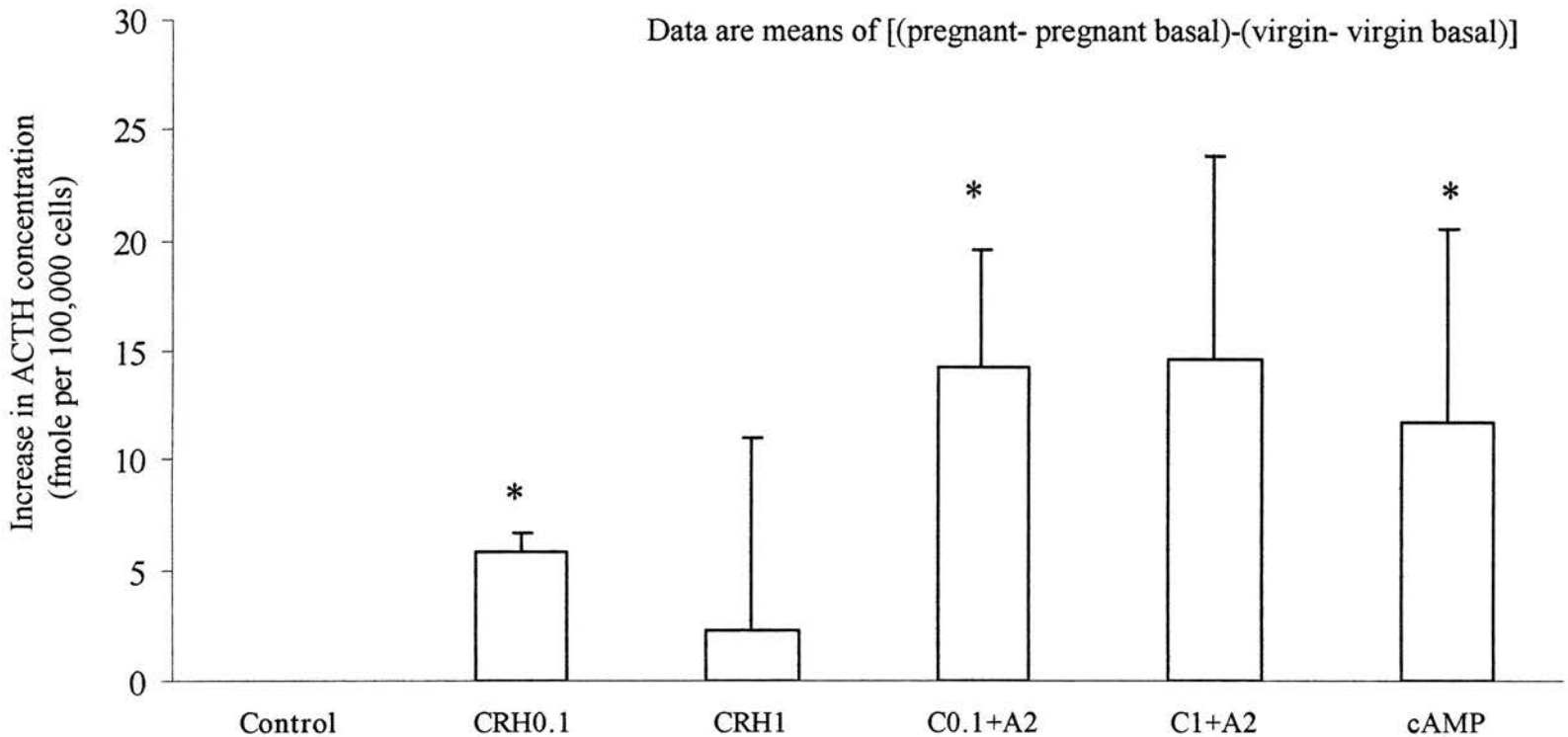


Figure 6.4 The effect of the secretagogues on ACTH secretion compared between the virgin and day 21 pregnant rats. The data in this histogram are the differences between day 21 pregnant and respective virgin delta values for each treatment, and this histogram is the summary of several independent individual measurements. Compared with the virgin group (control, value= 0), CRH at dose of 0.1 nM, the combination of 0.1 nM CRH and 2 nM vasopressin, and 8-CPT-cAMP in the pregnant group stimulated more ACTH secretion (*$p<0.05$, Kruskal-Wallis One-way ANOVA on Ranks and followed by Mann-Whitney Rank Sum Test). The combination of 1 nM CRH and 2 nM vasopressin increased ACTH secretion more in the pregnant group, but is not statistically significant. C 0.1= CRH 0.1 nM, n=4 experiments; C 1= CRH 1 nM, n=7 experiments; C 0.1+ V 2= CRH 0.1 nM + vasopressin 2 nM, n=4 experiments; C 1+V 2= CRH 1 nM + vasopressin 2 nM, n=4 experiments; cAMP=8-CPT-cAMP, n=7 experiments.

Comparing virgin rats, by the ratio calculation (Figure 6.5) or by calculating the differences between the pregnant delta values and the virgin delta values (Figure 6.6), secretion of ACTH by anterior pituitary cells from the day 10 pregnant rats were not significantly different in response to any secretagogue ($p>0.05$, Kruskal-Wallis One-way ANOVA on Ranks).

The total ACTH content in the anterior pituitary cells

The total ACTH contents were assayed from 200 µl frozen dispersed anterior pituitary cells. There was no difference in ACTH contents from 100,000 acutely dispersed anterior pituitary cells between virgin and either day 10 pregnant rats or day 21 pregnant rats (Figure 6.7, $p>0.05$, t-test).

The effectiveness of a V1a/b receptor antagonist, [dP(Tyr(Me)^2,Arg-NH$_2$^{9})AVP, on ACTH secretion from incubated acutely dispersed anterior pituitary cells

To investigate the *in vitro* effectiveness of the V1a/b receptor antagonist, [dP(Tyr(Me)^2,Arg-NH$_2$^{9})AVP, used in the stress study (Chapter 5), we measured ACTH secretion from acutely dispersed anterior pituitary cells by incubation with this V1a/b receptor antagonist added 5 min before the secretagogues. After 1 h incubation, vasopressin at 2 nM and CRH at 0.1 nM alone increased ACTH release compared with basal release, but the increases were not statistically significant (Figure 6.8; $p>0.05$, for vasopressin and CRH respectively, one-way ANOVA). Vasopressin potentiated the stimulatory effect of CRH on ACTH secretion, increasing ACTH release significantly ($p<0.05$, one-way ANOVA). As it is known from previous measurement that vasopressin at 2 nM does not have a significant effect on ACTH release from isolated anterior pituitary cells (this chapter), we did not test the effect of the V1a/b receptor antagonist on ACTH secretion directly by incubating this antagonist with vasopressin. The V1a/b receptor antagonist at the high dose (10^{-5} M) did not change the basal nor 0.1 nM CRH stimulated ACTH secretion ($p>0.05$, one-way ANOVA), but significantly reduced ACTH release stimulated by CRH plus vasopressin to the basal levels ($p<0.05$, one-way ANOVA). However, this V1a/b receptor antagonist at a low dose (10^{-7} M) did not affect the ACTH secretion stimulated by CRH plus vasopressin ($p>0.05$, one-way ANOVA).

Figure 6.5 Combined data from all experiments: pregnant day 10 versus virgin ratio

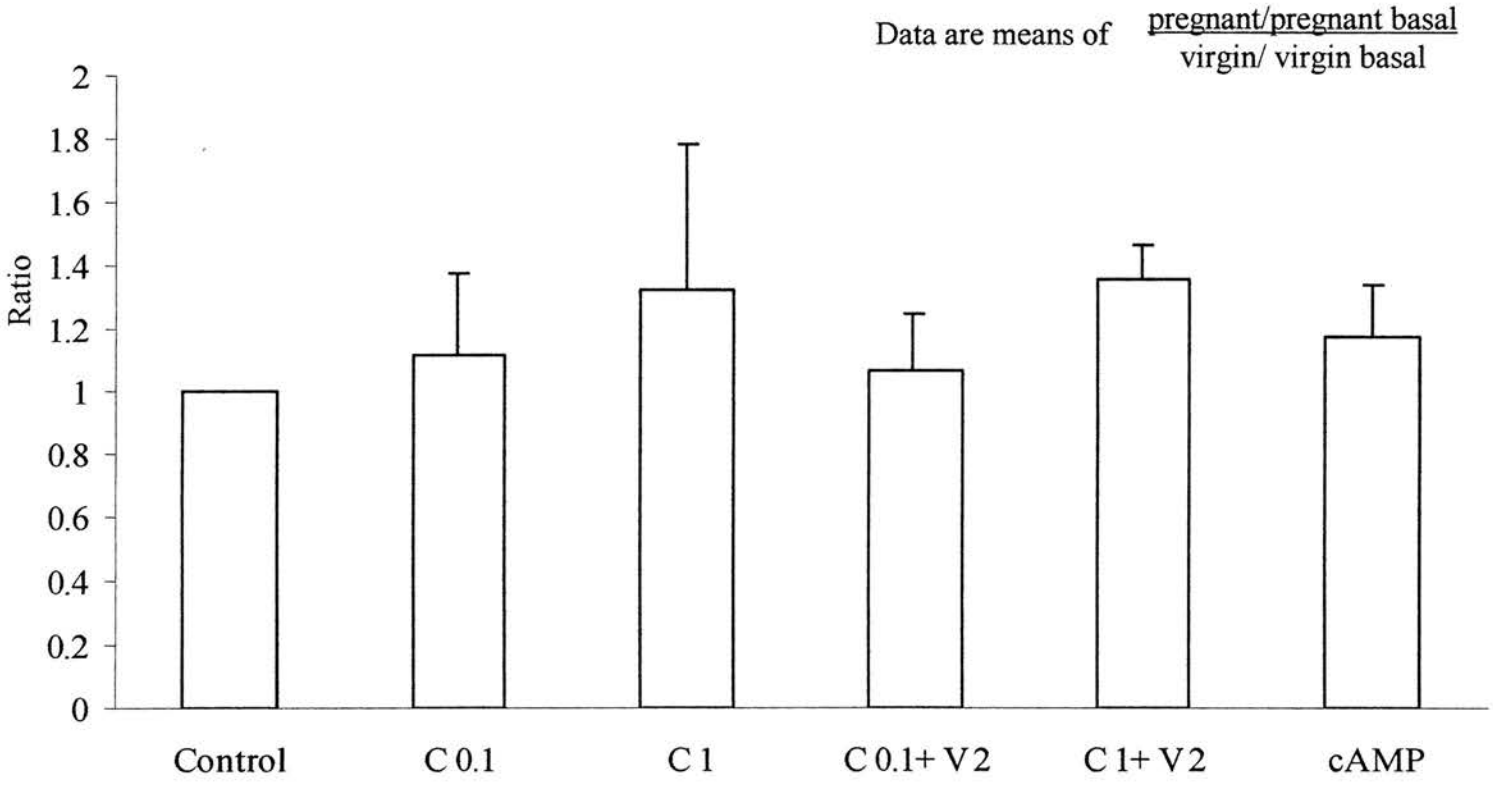


Figure 6.5 The effect of the secretagogues on ACTH secretion compared between the virgin and day 10 pregnant rats. The data in the histograms are means of ratio for pregnant versus virgin rats (based on respective ratio values). Compared with the virgin group (control, ratio= 1), there were no differences between the treatments and the control (Kruskal-Wallis One-way ANOVA on Ranks and followed by Mann-Whitney Rank Sum Test; C 0.1= CRH 0.1 nM, n= 3 experiments; C 1= CRH 1 nM, n= 3 experiments; C 0.1+V 2= CRH 0.1 nM + vasopressin 2 nM, n= 3 experiments; C 1+V 2= CRH 1 nM + vasopressin 2 nM, n= 3 experiments; cAMP=8-CPT-cAMP, n= 3 experiments.

Figure 6.6 Combined data from all experiments:
pregnant day 10 versus virgin increments

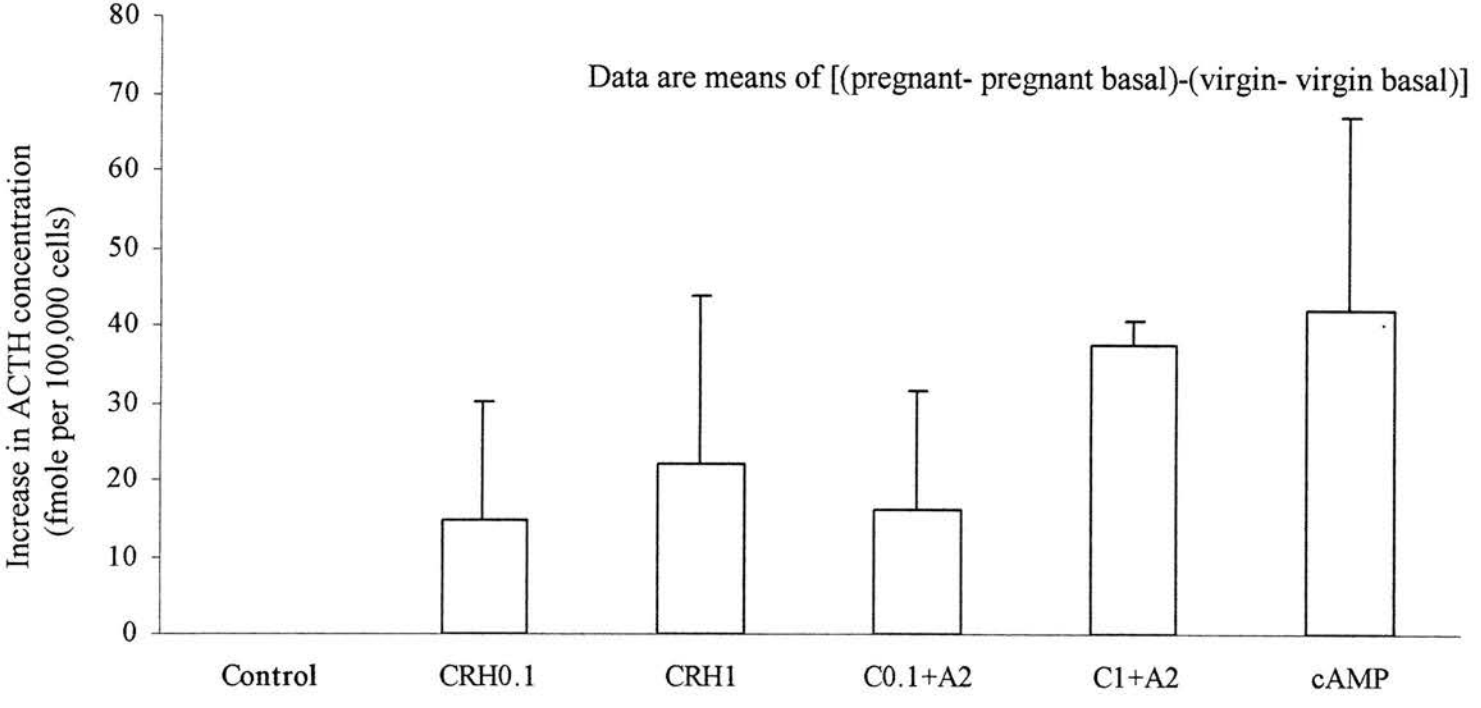


Figure 6.6 The effect of the secretagogues on ACTH secretion compared between the virgin and day 21 pregnant rats. The data in this histogram are the differences between day 10 pregnant and respective virgin delta values for each treatment, and this histogram is the summary of several independent individual measurements. Compared with the virgin group (control, value= 0), there were no differences between the treatments and the control (Kruskal-Wallis One-way ANOVA on Ranks and followed by Mann-Whitney Rank Sum Test; C 0.1= CRH 0.1 nM, n= 3 experiments; C 1= CRH 1 nM, n= 3 experiments; C 0.1+V 2= CRH 0.1 nM + vasopressin 2 nM, n= 3 experiments; C 1+V 2= CRH 1 nM + vasopressin 2 nM, n= 3 experiments; cAMP=8-CPT-cAMP, n= 3 experiments.

Figure 6.7 ACTH contents in the corticotrophs in virgin and pregnant rats

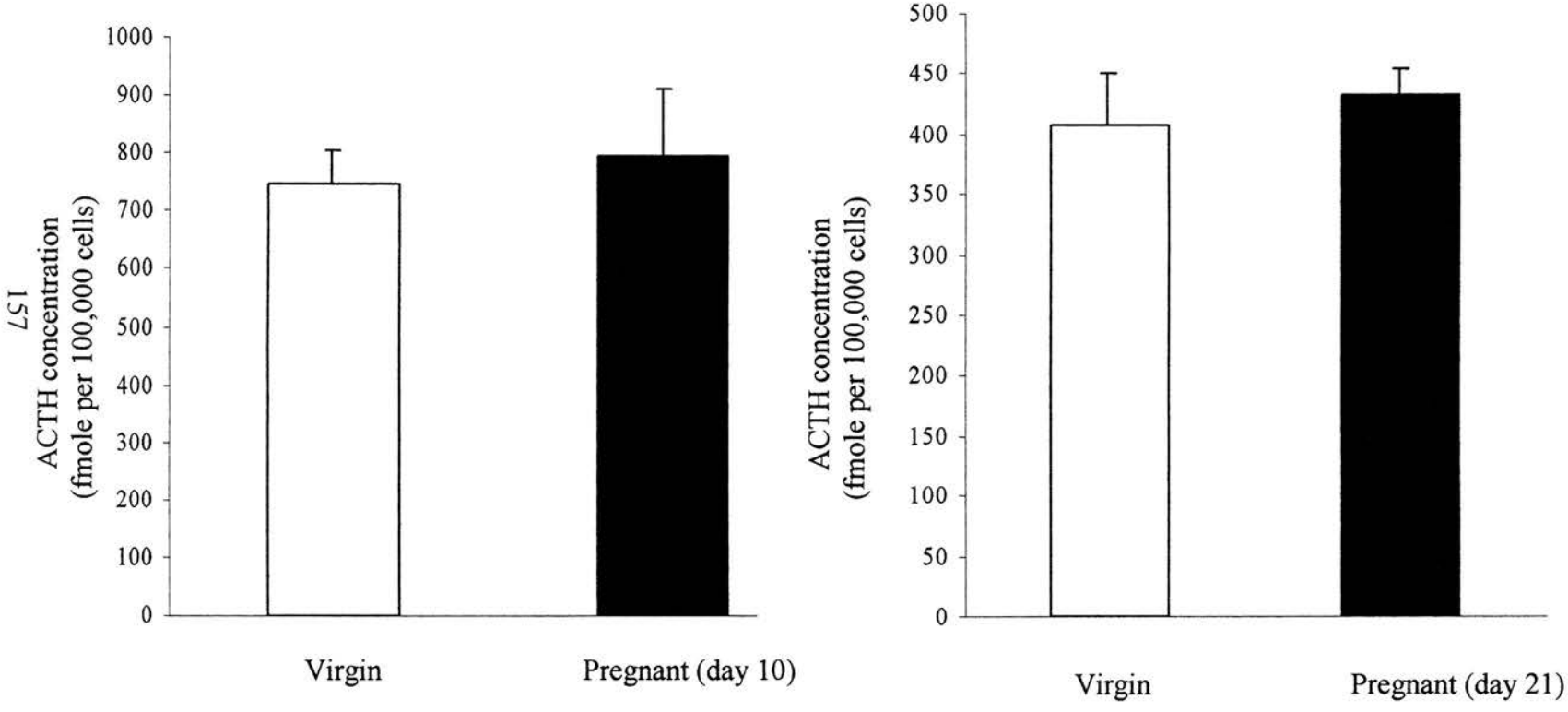


Figure 6.7 The total ACTH contents in anterior pituitary cells of the virgin and pregnant rats. Data are expressed as mean ± SEM. There were no differences of ACTH contents in the anterior pituitaries between the virgin and pregnant rats ($p>0.05$, t-test; $n=3$ for each group).

Figure 6.8 The effect of a V1a/b receptor antagonist, [dP(Tyr(Me)^2,Arg-NH$_2$^{9})AVP, on ACTH secretion from isolated anterior pituitary cells

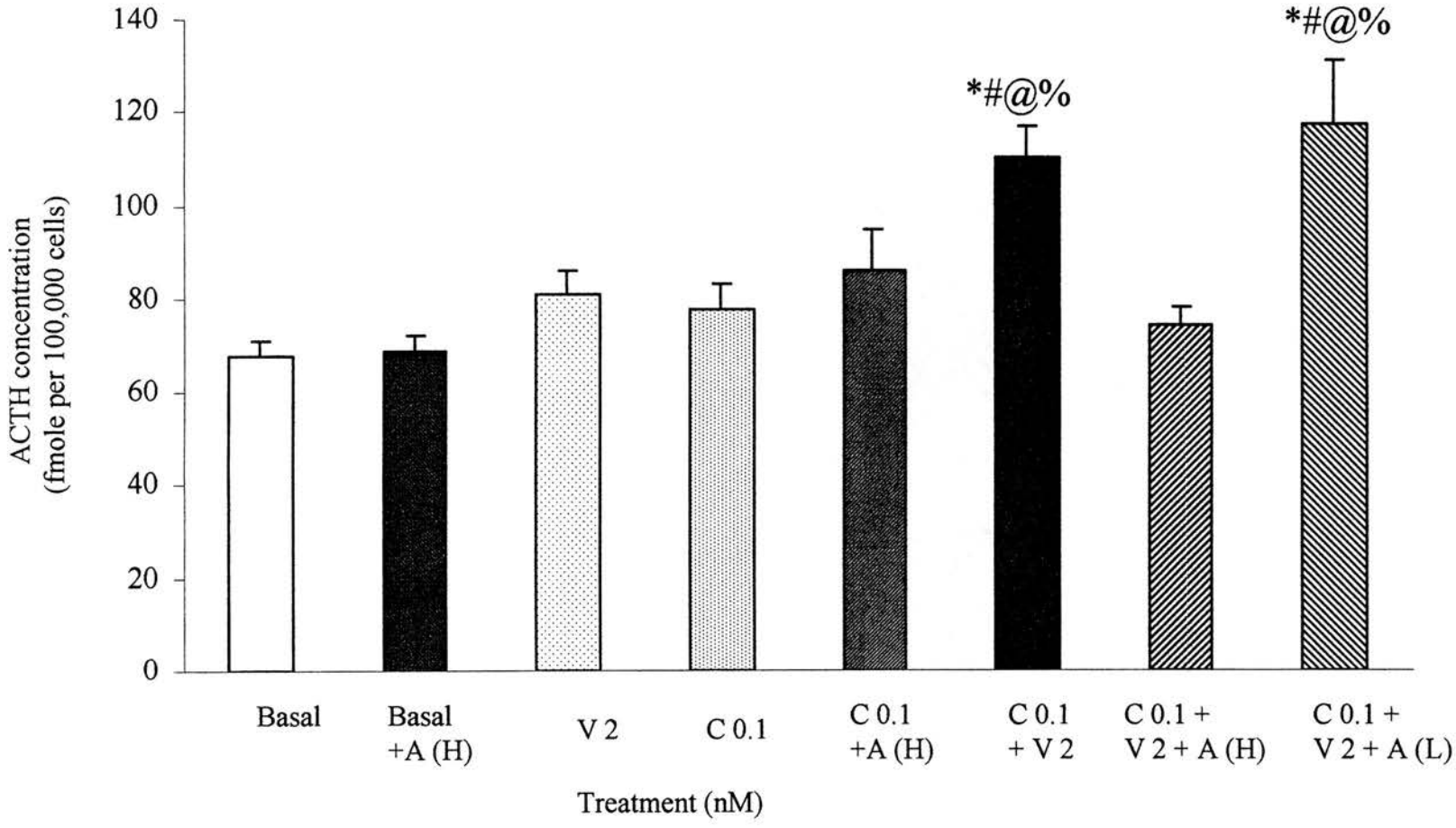


Figure 6.8 The effect of a V1a/b receptor antagonist, [dP(Tyr(Me)^2,Arg-NH$_2$^{9})AVP, on ACTH secretion from acutely isolated anterior pituitary cells. The data are the raw values expressed as mean ± SEM. The V1a/b receptor antagonist did not change basal, nor CRH- stimulated ACTH concentrations. However, at high dose (10^{-5} M), it significantly reduced the ACTH secretion stimulated by the combination 0.1 nM CRH and 2 nM vasopressin (*$p<0.05$ vs vehicle group, #$p<0.05$ vs C0.1+V2+A(H), @$p<0.05$ vs CRH 0.1, %$p<0.05$ vs V2, one-way ANOVA), but not at low dose (10^{-7} M). V 2 = vasopressin 2 nM; C 0.1= CRH 0.1 nM; A(H)= V1a/b receptor antagonist at a high dose (10^{-5} M); A(L)= V1a/b receptor antagonist at a low dose (10^{-7} M). N=6 in each group.

6.5 Discussion

The effects of secretagogues and cAMP on ACTH secretion by anterior pituitary cells

Consistent with previous findings (Gillies *et al.* 1982), the virgin rats showed a dose-dependent pattern of ACTH secretion from isolated anterior pituitary cells stimulated by CRH ranging from 0.01 to 10 nM. However, vasopressin alone at the doses we used (from 0.01 nM to 10 nM) did not significantly stimulate ACTH release. In this present study, we found the isolated anterior pituitary cells from the pregnant rats also showed a dose-dependent ACTH response to CRH, but not to vasopressin. Similarly, vasopressin potentiated the stimulatory effect of CRH on ACTH secretion from virgin and pregnant anterior pituitary cells. 8-CPT-cAMP, a membrane permeable cAMP analogue, also increased ACTH concentrations in virgin and pregnant rats. These results indicate that corticotrophs from pregnant rats do not have reduced responsiveness compared with virgin rats. Indeed, further analysis indicates increased responsiveness.

Increased effects of 0.1 nM CRH and 8-CPT-cAMP

The result from the present study showed that CRH at 0.1 nM produced a greater ACTH secretory response by the anterior pituitary cells from the day 21 pregnant rats than from the virgin rats (Figure 6.2, 6.3), whereas the number of CRH receptors on the corticotrophs, CRHR1, is reported to decrease in the pregnant rat (Neumann *et al.* 1998). So these data together suggest that enhanced post- receptor signalling and/or greater efficiency of the receptor recycling of CRHR1 mechanisms exist in pregnancy. The former hypothesis was supported by the results of the incubation with 8-CPT-cAMP. CRH stimulates the accumulation of cAMP in the corticotrophs (Labrie *et al.* 1982) and cAMP activates the PKA pathway leading to the release and also biosynthesis of ACTH. A cell membrane permeable cAMP analogue, 8-CPT-cAMP, resistant to hydrolysis by cyclic nucleotide phosphodiesterases was used in the present study to sustain a high level of intracellular cAMP. Anterior pituitary cells from day 21 pregnant rats released more ACTH in response to 8-CPT-cAMP, suggesting that the post receptor signalling is increased in pregnancy. This enhancement was not evident at day 10 of pregnancy, but this may be because the number of experiments at this stage was small. cAMP accumulations in anterior pituitary segments incubated with CRH were lower in pregnancy than in virgin

(Neumann *et al.* 1998), so the enhanced effect of cAMP in pregnancy may compensate for the reduced accumulation of cAMP stimulated by CRH. Increased CRHR1 efficiency may not be the explanation of the increased ACTH secretion in pregnancy *in vitro,* as cAMP accumulation is not increased from the anterior pituitary cells after incubation with CRH in pregnancy, on the contrary, it is reduced (Neumann *et al.* 1998). However, in the present study, the administration of 8-CPT-cAMP, which is resistant to hydrolysis by phosphodiesterase, prevented the degradation, so. the possibility that the degradation of cAMP by phosphodiesterase may increase in pregnancy cannot be ruled out, that is: cAMP degradation in pregnancy is enhanced, and the production of cAMP by the stimulation of CRHR1 is not changed or even increased, overall resulting in reduced cAMP accumulation.

The incubation of isolated anterior pituitary cells with the low dose of CRH (0.1 nM) showed a statistically significant greater release of ACTH in cells from day 21 pregnant rats than in virgins, by both ratio and increment calculations, but the effect of the high dose of CRH (1 nM) was significantly greater only by comparison of increments. It seems then that *in vitro*, without addition of vasopressin, CRH is more effective in stimulating ACTH secretion by corticotrophs from pregnant rats. This suggests that the reduced effect of CRH *in vivo* in pregnancy may be due to reduced secretion of another secretagogue, such as vasopressin (see Chapter 5), rather than to reduced effectiveness of CRH alone.

Increased effect of 0.1 nM CRH and 2 nM vasopressin

By receptor binding studies, CRH and vasopressin receptors in the anterior pituitary are reduced in pregnancy (Toufexis *et al.* 1999), indicating a mechanism for possible reduced corticotroph sensitivity to secretagogues. However, ACTH release from acutely dispersed anterior pituitary cells incubated for 1 h with CRH plus vasopressin was greater in pregnancy. This discrepancy may be due to faster turnover of these two receptors in pregnancy, associated with increased efficiency. This increased secretion in response to the combination of secretagogues may not reflect differences in number of CRH or V1b receptors, or in the expression of V1b mRNA, but rather a change in post receptor signalling.

Vasopressin stimulates ACTH secretion through activation of PKC with subsequent generation of IP_3 and diacylglycerol. IP_3 is believed to cause the release of calcium from intracellular stores, and diacylglycerol acts in concert with calcium to translocate PKC from cytosolic sites to the plasma membrane coincident with enzyme activation (King & Baertschi 1990). Vasopressin potentiates the effect of CRH on ACTH secretion by a synergistic increase in intracellular cAMP accumulation in the corticotrophs (Giguere & Labrie 1982). The potentiating action of vasopressin on CRH stimulation of ACTH secretion involves increase in cAMP formation by stimulation of adenylate cyclase (Abou-Samra *et al.* 1987) and an decrease in cAMP degradation by inhibiting phosphodiesterase activity (Giguere & Labrie 1982), increasing intracellular cAMP accummulation, leading to potentiated ACTH secretion. In the present study, ACTH secretion stimulated by CRH plus vasopressin was greater in pregnancy. However, the ratio (R2) of ACTH release for the pregnant versus virgin groups was not different ($p>0.05$, one way ANOVA) between CRH treatment alone (1.37, 1.25 for the 0.1 nM and 1 nM CRH treatment respectively) and CRH plus vasopressin (1.60, 1.54 for vasopressin plus 0.1 nM and 1 nM CRH treatment respectively), so it is unlikely the potentiation mechanism by vasopressin acts is enhanced in pregnancy *in vitro*, and the greater ACTH secretion is likely to be mainly due to the increased CRH effect through enhanced cAMP signalling.

The combination of CRH (200 ng/kg) and vasopressin (1.7 μg/kg) treatment by intravenous injection increased plasma ACTH concentrations similarly in virgin and pregnant rats (see Chapter 5). This is similar to lack of a reduction *in vitro* in the combined effects of CRH and vasopressin. However, the greater effect of CRH alone *in vitro* was not seen *in vivo*: this may be because the *in vivo* dose did not compensate for a likely lack of vasopressin secreted by the parvocellular CRH/vasopressin neurones in pregnancy.

There were no differences in ACTH secretion stimulated by any secretagogues between day 10 pregnant rats and virgin rats, suggesting that corticotroph cellular signalling and sensitivity are not altered in early pregnancy. This is consistent with the POMC mRNA expression (see Chapter 3) and ACTH response to swim stress studies (Neumann *et al.* 1998).

ACTH contents

ACTH belongs to a family of peptides derived from a common precursor protein encoded by the POMC gene (Chretien & Seidah 1981; King & Baertschi 1990; Lowry *et al.* 1980). Although POMC mRNA expression in the anterior pituitary was reduced in pregnancy (Chapter 3), the ACTH contents in the anterior pituitary measured in the present study were not different between virgin and pregnant rats, suggesting the processes of the POMC gene transcription and the precursor protein modification is faster, and/or the degradation of ACTH is slower in pregnancy.

The diurnal pattern of ACTH secretion disappears, the afternoon peak lost, so the secretion of ACTH per 24 h near the end of pregnancy is decreased (Atkinson & Waddell 1995). In the present study, total ACTH contents in the corticotrophs were far more than required for secretion after incubation with secretagogues (about 5-10 times higher than basal secretion and 3-6 times higher than incubation with 0.1 nM CRH and 2 nM vasopressin), so the reduced ACTH secretion in response to acute stress or CRH, or circadian basal release *in vivo* is mainly due to decreased release from the corticotrophs.

V1b receptor antagonist

The effectiveness of the V1b receptor antagonist we used *in vivo* (see Chapter 5) on ACTH secretion *in* vitro from acutely dispersed anterior pituitary cells from virgin rats was investigated. This antagonist decreased ACTH secretion *in vivo* (Chapter 5). In the present study, the V1a/b receptor antagonist did not affect basal secretion. This antagonist decreased the stimulation of ACTH secretion by CRH plus vasopressin incubation, as this antagonist did not change CRH-induced ACTH secretion, indicating it does not interact with CRHR1, so its antagonistic effect was via the V1b receptors on the corticotrophs, blocking the potentiation effects of vasopressin.

In summary, the present study showed a dose-dependent effect of CRH *in vitro*, and the potentiation by vasopressin of CRH action on ACTH secretion, by corticotrophs from pregnant rats. The finding that corticotrophs from day 21 pregnant rats released more ACTH when incubated with a low dose (0.1 nM) of CRH, and with CRH plus vasopressin, and showed a greater effect of a cAMP analogue suggests changes in post receptor signalling mechanisms in pregnancy.

Chapter 7

Effect of neurosteroids on the ACTH response to stress during pregnancy

7.1 Introduction

It is well established that plasma progesterone levels increase progressively during pregnancy in humans and rats, and reach their highest levels one or two weeks before delivery in humans (Mathur *et al.* 1980) or 1-2 days before delivery in rats (Concas *et al.* 1999). The neuroactive steroids, allopregnanolone and THDOC (the metabolites of progesterone) concentrations are also at the peak values prior to parturition in the brain (Concas *et al.* 1999). Both brain and plasma allopregnanolone and THDOC concentrations are also elevated after acute stress or ACTH infusion (Schambelan & Biglieri 1972), which are released from the adrenal gland (Purdy *et al.* 1991). These neurosteroids exert an important inhibitory effect on the HPA system by interacting with $GABA_A$ receptor in the brain (Breier *et al.* 1992; Owens *et al.* 1992; Patchev *et al.* 1994; Patchev *et al.* 1996; Bernet *et al.* 2000; Cullinan 2000). So it is postulated that these neurosteroids may participate in the stress response as a homeostatic factor to terminate the response (Jessop 1999). This mechanism may be crucial for the attenuated ACTH response to stress at the late stage of pregnancy.

Allopregnanolone is a 5α-reduced metabolite of progesterone and acts as a major $GABA_A$ receptor allosteric modifier by increasing the duration and frequency of GABA-stimulated chloride channel openings (Majewska *et al.* 1986; Lan *et al.* 1991; Paul & Purdy 1992). THDOC, another 5α-reduced derivative from progesterone via deoxycorticosterone (DOC), is also a potent $GABA_A$ receptor active neurosteroid (Schneider & Honour 1992; Winkel *et al.* 1980), but the quantity of THDOC produced through this pathway is small compared with the synthesis of allopregnanolone, so it is unlikely that THDOC is as important as allopregnanolone.

In the brain these neurosteroids are synthesised by oligodendrocytes and glial cells (Baulieu & Robel 1990).

Neurosteroid concentrations in plasma and extracts from brain are markedly increased shortly after application of different stress paradigms (Purdy *et al.* 1991; Barbaccia *et al.* 1994; Barbaccia *et al.* 1998). In particular, during the stress response brain content of allopregnanolone reaches a high value (Korneyev *et al.* 1993; Purdy *et al.* 1991). Allopregnanolone can attenuate the activity of the HPA axis challenged by various stresses (Guo *et al.* 1995; Patchev *et al.* 1996; Bernet *et al.* 2000). THDOC is also reported to be able to attenuate mild stress-induced increases in plasma corticosterone concentrations via GABAergic mechanisms (Owens *et al.* 1992). These data indicate that neurosteroids such as allopregnanolone and THDOC are part of the negative feedback mechanism in the stress response.

The conversion of progesterone into allopregnanolone and THDOC is catalysed by 5α-reductase irreversibly (see Chapter 1.5.2) (Abul-Hajj 1972; Winkel *et al.* 1980; Schneider & Honour 1992). Two types of 5α-reductase, type I (Andersson & Russell 1990) and type II (Andersson *et al.* 1991), have been isolated and identified from human and rat. 5α-reductase is widely expressed in mammalian tissues (Li *et al.* 1995) and the brain is a rich source of this enzyme (Melcangi *et al.* 1993). The enzyme required for converting dihydroprogesterone (the reduced form of progesterone) to allopregnanolone, 3α-hydroxysteroid dehydrogenase (3α-hydroxysteroid oxidoreductase), is also broadly distributed in the brain (Li *et al.* 1997). 17β-N,N-diethylcarbomyl-4-aza-4-methyl-5α-androstan-3-one (4-MA) is a potent competitive inhibitor of type I α- reductase and a less potent inhibitor of type II α- reductase (Li *et al.* 1995). 4-MA inhibits 5α-reductase activity in many tissues including the brain and peripheral tissues (Bitran *et al.* 1995; Mellin *et al.* 1993).

7.2 Aims

As neurosteroids can attenuate the activity of the HPA axis and brain levels increase during the late stage of pregnancy, we hypothesized that neurosteroids may attenuate

HPA axis activity during pregnancy. To investigate possible roles of neurosteroids in the late stage of pregnancy in attenuating stress responses of the HPA axis, we blocked conversion of progesterone to allopregnanolone and THDOC with a competitive 5α- reductase inhibitor, 4-MA.

7.3 Materials and methods

5α-reductase antagonist, 4-MA, and stress-stimulated ACTH secretion

To study neurosteroid actions on ACTH secretion, individually caged pregnant and virgin rats had a silastic jugular cannula implanted by the method described in detail in Chapter 2.1.2.1. The experiment was carried out four to five days following surgery to allow the animals to recover. 4-MA (a kind gift from Prof. Ian Mason, University of Edinburgh) was dissolved in sesame oil (90% v/v, in absolute alcohol). It has been reported that 4-MA administration (33 mg/kg, 2 h before) blocks the anxiolytic effect induced by progesterone. Furthermore, 4-MA blocks the production of allopregnanolone on administration of exogenous progesterone (Bitran *et al.* 1995). In the present study, we adopted a similar dose and time course with minor modification by giving an extra dose of 4-MA (33 mg/kg) 18 h before the experiment. At 16:00 h on the day before experiment, the rats were given 4-MA (33 mg/kg), or vehicle (10% ethanol and 90% sesame oil, 660 μl/kg) subcutaneously. On the day of experiment (day 21 of pregnancy), the rats were administered the same dose of 4-MA or vehicle at 08:00 h in the following morning. The jugular cannula was then flushed and connected to a syringe filled with sterile heparinised saline. Two basal blood samples (0.4 ml) were taken 60 min and 90 min after the second subcutaneous injection. All the rats were forced to swim for 90 s in deep water (19°C), and further blood samples were taken after 5, 15, 30 and 70 min. Each blood sample was collected into an Eppendorf tube containing 15 μl 5% EDTA per 100 μl blood. Blood was replaced with 0.9% saline. Blood samples were cooled on ice, centrifuged at 12,000 g for 5 min and plasma separated and stored at -70°C until assayed.

4-MA and exogenous CRH-induced ACTH secretion

Immediately after the 70 min sample in the same experiment above, each rat received 200 ng/kg CRH intravenously, to examine the effect of 4-MA on pituitary responses

to exogenous CRH, and a further blood sample taken after 10 min. Each blood sample was collected into an Eppendorf tube containing 15 µl 5% EDTA per 100 µl blood. Blood was replaced with 0.9% saline. Blood samples were cooled on ice, centrifuged at 12,000 g for 5 min and plasma separated and stored at -70°C until assayed. Plasma ACTH concentrations were assayed by RIA with the ACTH kits from ICN (details in Chapter 2.2.2.5)

All rats were killed by decapitation, the number of fetuses counted and fetal viability was assessed.

Statistics

The effect of 4-MA on the ACTH response to stress were analysed by two-way ANOVA for repeated measures followed by post-hoc Newman-Keuls tests. Data from the effects of CRH stimulated ACTH secretion after the stress study were analysed by one-way ANOVA and post-hoc Student-Newman-Keuls tests.

7.4 Results

Effect of 4-MA on the ACTH response to stress

One and half hours after the second 4-MA administration, the basal ACTH concentrations before swim stress did not differ between groups (Figure 7.1, $p>0.05$, two-way ANOVA for repeated measures). Following swim stress the ACTH plasma concentrations were significantly increased in all groups at 5, 15 and 30 min (virgin vehicle, n=6; virgin 4-MA, n=7; pregnant vehicle, n=7; pregnant 4-MA, n=9. $p<0.05$). The peak ACTH response occurred at 5 min after the stress in all groups. Plasma ACTH concentration was lower in response to swim stress in vehicle-treated pregnant rats compared to vehicle-treated virgins at the 5-min time point ($p<0.05$). 4-MA treatment increased plasma ACTH concentrations 5 min after swimming in the pregnant group, but not in the virgins, compared to the respective vehicle-treated groups ($p<0.05$). Plasma ACTH concentrations remained at high levels in both the 4-MA treated virgin and pregnant groups compared with the vehicle groups at 15 and 30 min after stress, although there were no statistically significant differences. Plasma

ACTH concentrations returned close to the basal levels by 70 min post swim stress in both the virgin and pregnant vehicle-treated groups, but remained significantly higher in the 4-MA treated groups ($p<0.05$).

The histograms (Figure 7.2) present the changes (delta) in plasma ACTH concentrations at 5, 15, 30 and 70 min post swim stress relative to the pre-swim basal concentrations. 4-MA increased the ACTH response to swim stress only in the pregnant rats at the 5 min time point, and in both pregnant and virgin rats at 70 min ($p<0.05$, two-way ANOVA for repeated measures).

4-MA and exogenous CRH-induced ACTH secretion

All groups were intravenously injected with CRH 200 ng/kg at 70 min after the swim stress. Data presented as actual plasma ACTH concentrations and ACTH increments are in Figure 7.3 and Figure 7.4 respectively. Ten min after this dose of CRH, ACTH concentration was greater in 4-MA treated both virgin and pregnant rats compared to respective vehicle treated rats (Figure 7.3, $p<0.05$, two-way ANOVA for repeated measures). ACTH concentrations were also greater in the vehicle treated virgin rats than those in the vehicle treated pregnant rats (Figure 7.3, $p<0.05$). The elevation of plasma ACTH concentrations in vehicle-treated virgins was significantly greater than that in the vehicle-treated pregnant rats (Figure 7.4, $p<0.05$, one-way ANOVA for repeated measures). 4-MA treatment did not change ACTH increment in response to exogenous CRH in virgins or in pregnant rats (Figure 7.4, $p>0.05$). There was no difference in ACTH increment after CRH stimulation between 4-MA- treated virgin and pregnant rats (Figure 7.4). However, because samples could only be obtained from 3 pregnant rats treated with 4-MA and CRH, and the increments in ACTH concentrations in these rats were small, there were no significant differences between this and the other groups when increments were compared (Figure 7.4).

Fetal viability was checked after the experiment. The fetuses (about 10-15 fetus per pregnant rat) were alive and moving in both the 4-MA treatment group and the vehicle group, with no placental detachment or intrauterine bleeding; however, uterine blood vessels appeared more distended in the 4-MA treated pregnant rats.

Figure 7.1 Effects of a 5α- reductase inhibitor, 4- MA, on stress- stimulated ACTH release during pregnancy

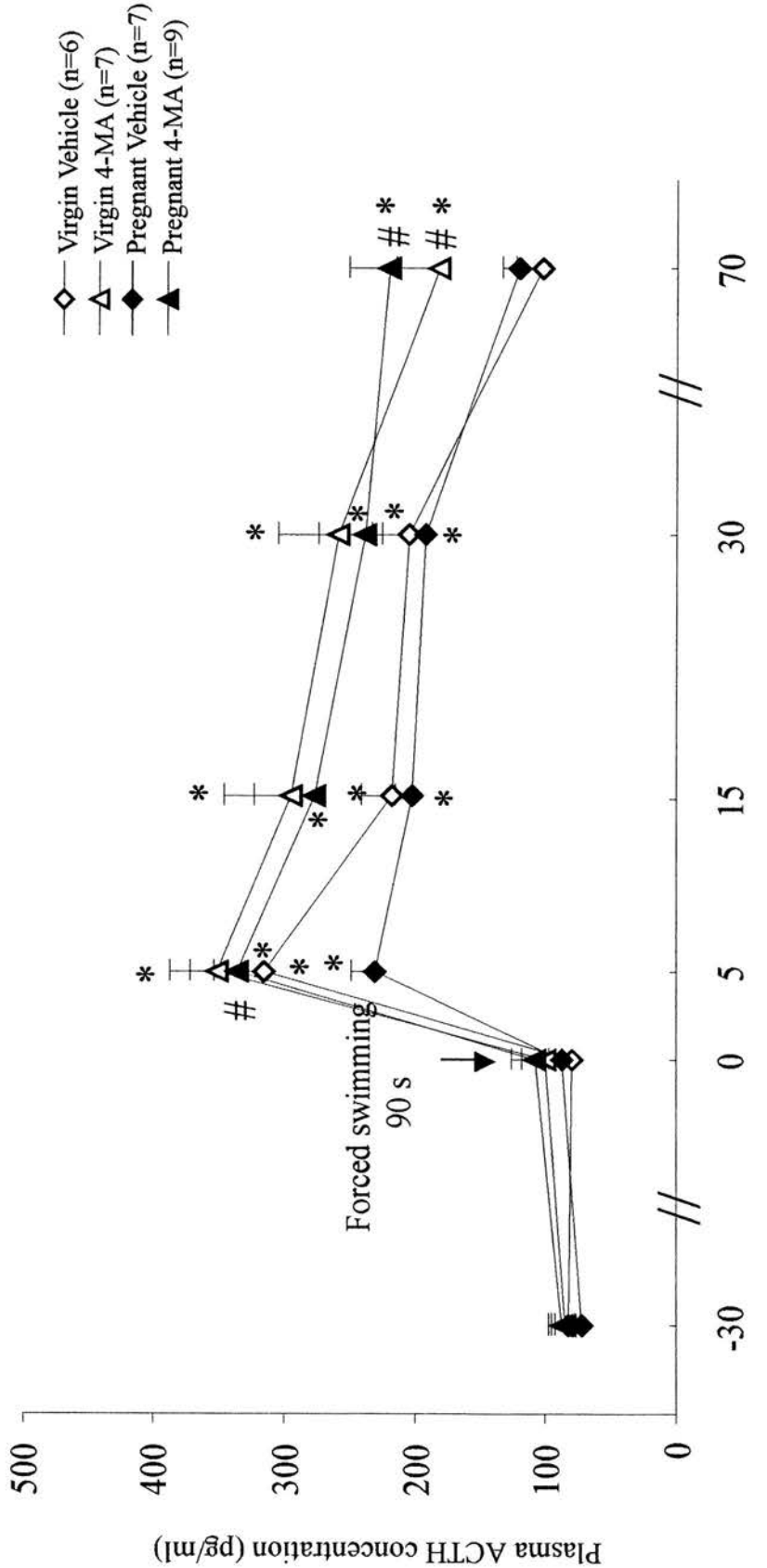


Figure 7.1 The effect of 4-MA on plasma ACTH concentrations in pregnant (day 21) and virgin rats. 4-MA (33 mg/kg) or vehicle was injected at 18 h and 2 h before blood sampling. Data are mean ± SEM. Basal blood samples were collected from a jugular vein 30 min and immediately before forced swimming (90 s, 19°C). Then further blood samples were taken 5, 15, 30 and 60 min after forced swimming. Values are means SEM. Statistical analysis: ACTH concentrations at 5 min in the 4-MA treated pregnant group were significantly higher than in the vehicle treated pregnant group ($p<0.05$). 70 min post swim stress, ACTH levels remained significantly higher in the 4-MA- treated virgin and pregnant groups ($p<0.05$) compared to respective vehicle-treated groups. *$P<0.05$ *vs* basal, #$p<0.05$ *vs* respective vehicle group, $p<0.05$ *vs* respective vehicle virgin groups, two-way ANOVA for repeated measurements followed by post-hoc Student-Newman-Keuls tests. .

Figure 7.2 Increments in ACTH concentrations after forced swimming in virgin and pregnant rats

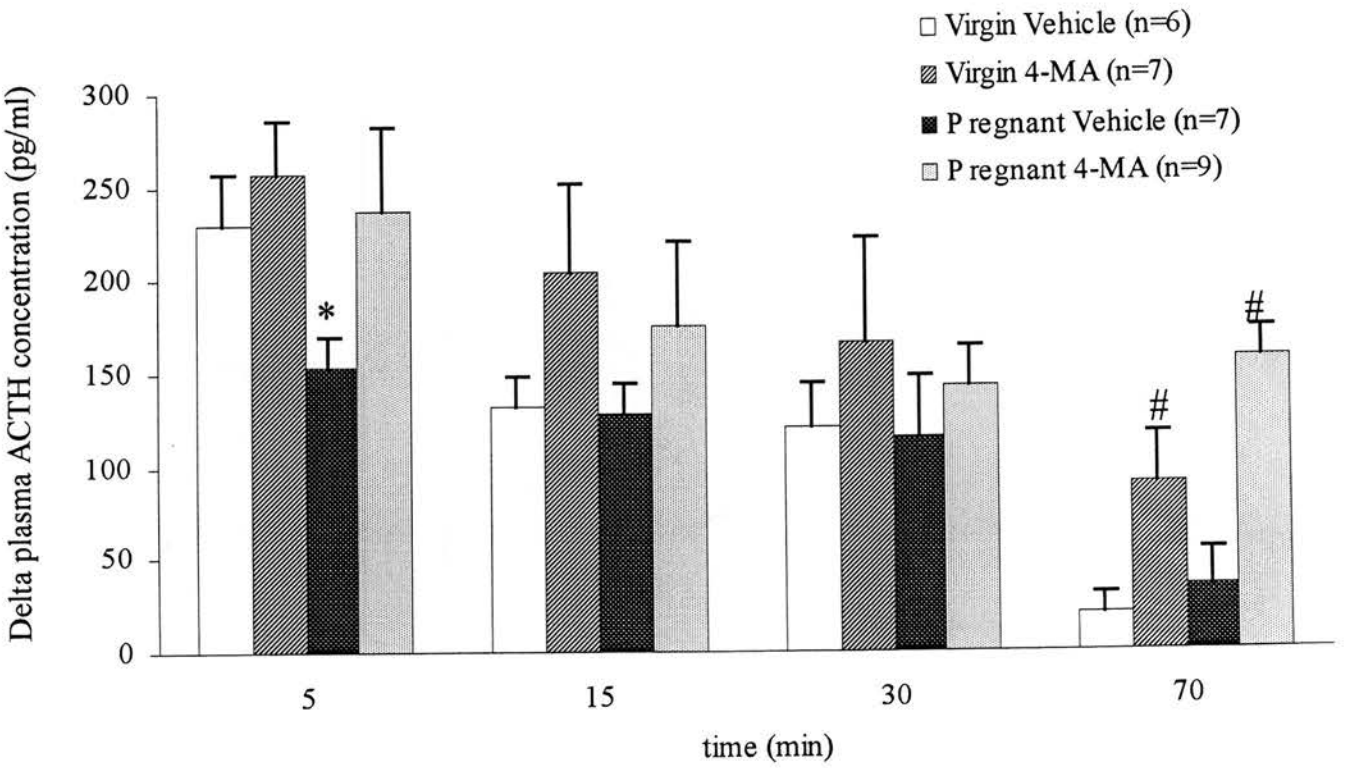


Figure 7.2 The effect of 4-MA on plasma ACTH concentrations in pregnant (day 21) and virgin rats. Histograms of the changes from basal of ACTH concentrations after forced swimming. Data are mean± SEM. Details in figure 7.1. * $p<0.05$ *vs* all other groups at the same time, two-way ANOVA; #$p<0.05$ *vs* respective vehicle control groups.

Figure 7.3 Effects of a 5α- reductase inhibitor, 4- MA, on CRH stimulated ACTH release during pregnancy

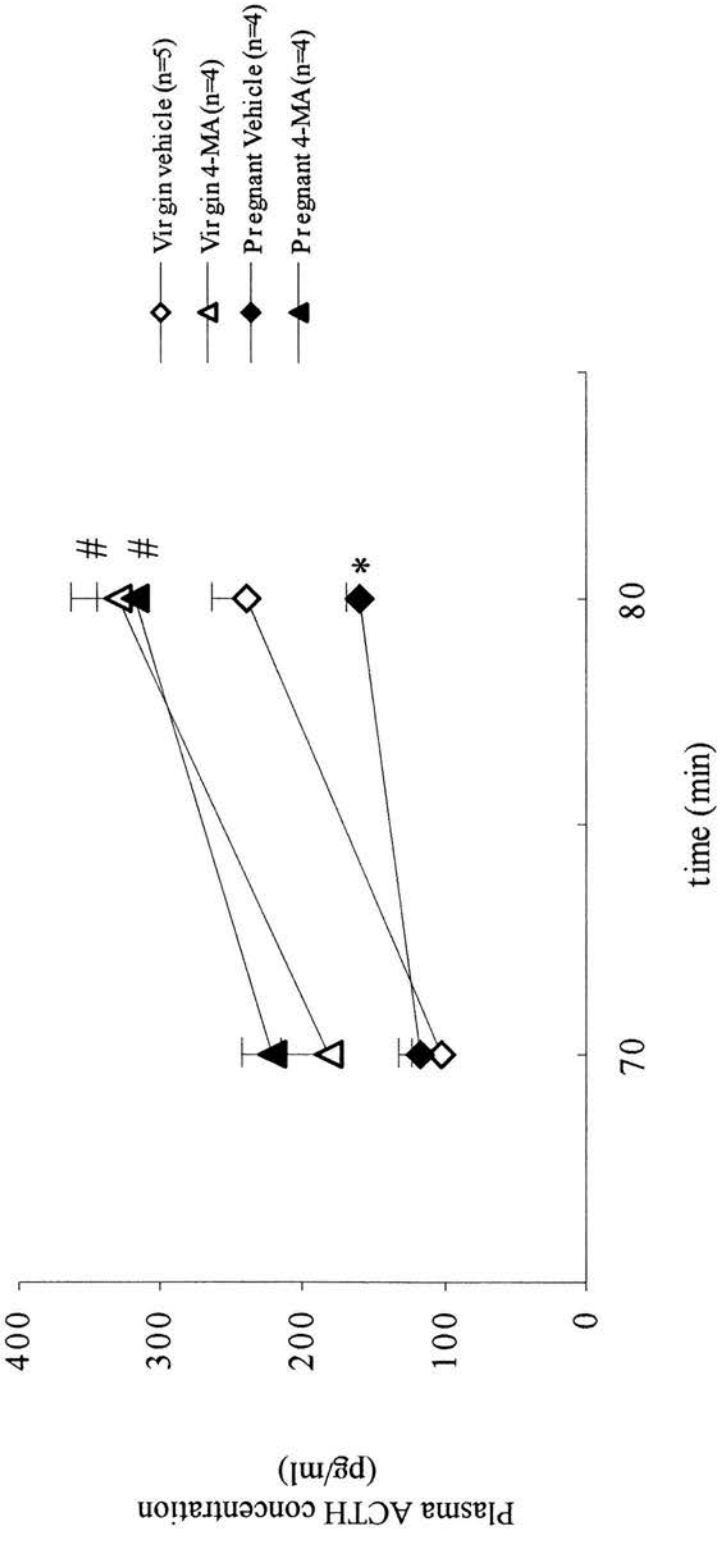


Figure 7.3 ACTH secretion stimulated by CRH after 4-MA injection. Each rat received 200 ng/kg CRH intravenously after the 70 min post- stress sample, and further blood samples were taken 10 min later. ACTH concentrations were greater in the virgin vehicle group than those in the day 21 pregnant vehicle group (*$p<0.05$ vs virgin vehicle, #$p<0.05$ vs pregnant vehicle, two-way ANOVA). ACTH levels in the 4-MA treated group were higher than those in the day 21 pregnant 4-MA treated group, but is not significant different. VV=virgin vehicle (n=5); VM=virgin 4-MA treated group (n=4); PV=pregnant vehicle group (n=4); PM=pregnant 4-MA treated group (n=4).

Figure 7.4 Increments in ACTH secretion stimulated by CRH after 4-MA administration in virgin and pregnant rats

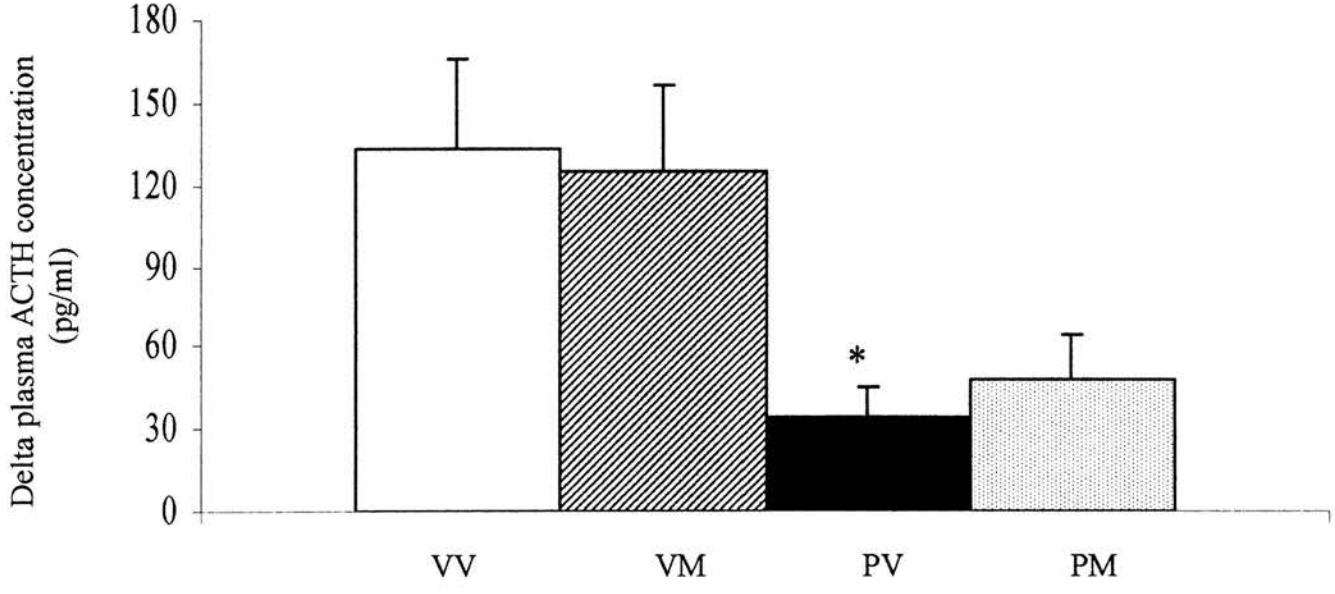


Figure 7.4 ACTH secretion stimulated by CRH after 4-MA injection expressed as histograms. Data are mean± SEM. Details in figure 7.3. The increments were calculated compared with 70 min basal ACTH concentrations. Increases in ACTH concentrations were greater in the virgin vehicle group than those in the day 21 pregnant vehicle group (*$p<0.05$, one-way ANOVA). Increases in ACTH concentrations in the 4-MA treated virgin group were higher than those in the pregnant 4-MA treated group, but not significantly different. VV=virgin vehicle (n=5); VM=virgin 4-MA treated group (n=4); PV=pregnant vehicle group (n=4); PM=pregnant 4-MA treated group (n=3).

7.5 Discussion

Effect of 4-MA on the ACTH response to stress

Allopregnanolone is a neuroactive steroid involved in modulating behavioural functions, stress responses, and the neuroendocrine axis in rats. Changes in plasma allopregnanolone levels throughout the menstrual cycle have been reported in healthy women (Schmidt *et al.* 1994; Wang *et al.* 1996) and in the rat brain during the estrous cycle (Palumbo *et al.* 1995). Previous findings suggest progesterone may exert its inhibitory effect on the HPA axis system following metabolism to neurosteroids and subsequent interaction with $GABA_A$ receptors (Majewska *et al.* 1986; Jessop 1999). However, progesterone receptor has not been detected in the PVN by either progesterone binding study (Sar 1988) or a progesterone receptor an immunohistochemistry study (Fenelon & Herbison 2000). Thus it is unlikely that progesterone has a role in the function of PVN neurones through genomic actions directly. Subcutaneous 4-MA administration increased ACTH secretion in response to swim stress in pregnant rats, which suggests that progesterone metabolites may contribute to the regulation of HPA activation.

It is well established that allopregnanolone and THDOC are powerful allosteric modulators of the $GABA_A$ receptor (Lambert *et al.* 1995). 4-MA inhibits 5α reductase activity, thus blocking the conversion of progesterone to allopregnanolone. 4-MA has been reported to reduce progesterone anti-seizure actions in the absence of estrogen (Frye 1995). In ovariectomised rats, the anxiolytic effect observed (increasing proportion of time spent on the open arms in the plus- maze test and reducing the duration of burying behaviour in the defensive burying test) after progesterone administration is prevented by 4-MA pre-treatment (Bitran *et al.* 1995). 4-MA blocked the production of allopregnanolone in the plasma from exogenous progesterone (Bitran *et al.* 1995). The effect of 4-MA on behaviour is paralleled by a block of the potentiating effect of progesterone administration on the GABA-gated Cl^- channel response in cortical synapatoneurosomes from ovariectomised rats, which suggests that 4-MA at this dose and time course reduced allopregnanolone levels in the brain. 4-MA will also block the production of THDOC, another $GABA_A$ receptor active neurosteroid, which is derived from progesterone via DOC catalysed by 5α reductase (Abul-Hajj 1972; Schneider & Honour 1992; Winkel *et al.* 1980).

In rats, plasma progesterone levels are increased throughout pregnancy reaching a peak at around day 16-20 when they are about 10-fold greater than the estrus values, then returning to the control values at day 22, just before parturition. Cerebrocortical progesterone levels change with a similar pattern to plasma concentrations during pregnancy (Concas *et al.* 1999). The plasma and cerebrocortical concentrations of the metabolites of progesterone, allopregnanolone and THDOC, are at their highest levels around day 20 of pregnancy (Concas *et al.* 1999) and are still at high levels compared with the virgins on day 21 of pregnancy (Frye & Bayon 1998).

Although progesterone has been considered only as a female reproductive hormone, substantial evidence has been reported that stress can increase progesterone levels in the plasma (Deis *et al.* 1989; Nequin *et al.* 1975; Purdy *et al.* 1991; Schaeffer & Aron 1987). Elevated levels of plasma progesterone accompany the increase in corticosterone after stress in male rats (Nequin *et al.* 1975; Purdy *et al.* 1991; Schaeffer & Aron 1987). Stress induced progesterone secretion in male and female rats is derived from the adrenal gland, because the response is abolished after adrenalectomy (Deis *et al.* 1989; Nequin *et al.* 1975; Purdy *et al.* 1991).

The physiological role of this progesterone in the HPA axis is not clear, but it is well established that the circulating progesterone can enter the brain, providing allopregnanolone to interact with $GABA_A$ receptors. Thus progesterone of adrenal origin is also considered as a precursor of allopregnanolone in the brain. Brain tissues can also produce progesterone and its metabolites after stress (Barbaccia *et al.* 1998).

In pregnancy, allopregnanolone has enhanced actions on $GABA_A$ receptors on oxytocin neurones at the end of pregnancy. The cellular levels of $GABA_A$ receptor $\alpha 1$ subunit mRNA are elevated with the progression of pregnancy in oxytocin neurones of the SON and PVN and are reduced about 30-40% in the last two days before pregnancy (Fenelon & Herbison 1996). In ovariectomised female rats, a progesterone implant for 9 days increased GABA receptor $\alpha 1$ subunit mRNA expression both in vasopressin and oxytocin rich PVN and SON neurones (Fenelon & Herbison 2000). The increased $GABA_A$ receptor $\alpha 1$ subunit expression and cerebral levels of

allopregnanolone together may be important for the attenuated HPA axis response to stress during pregnancy, if similar changes occur in the GABAergic mechanisms regulating PVN CRH/vasopressin neurones in pregnancy. However, nothing is presently known about this.

It is well known from *in vivo* studies that GABAergic mechanisms are involved in inhibiting the HPA axis (Makara & Stark 1974) and that the hypothalamus is a major site of action as $GABA_A$ receptors and a dense network of glutamate decarboxylase (GABA synthesising enzyme)-positive nerve fibres are present in the hypothalamus. Secretion of CRH, the major stimulator of the HPA axis, is negatively regulated by the GABAergic system. GABA is detected in the CRH-containing parvocellular neurones in the PVN (Meister *et al.* 1988), and potentially influences CRH expression via GABAergic neurones within the bed nucleus of the stria terminalis and preoptic nuclei which project into the PVN (Herman & Cullinan 1997). A $GABA_A$ receptor agonist inhibits CRH secretion from the hypothalamus *in vitro* (Calogero *et al.* 1988) and CRH secretion from the hypothalamic PVN into the hypophysial-portal circulation (Plotsky *et al.* 1987). Vasopressin is also believed to be inhibited by the GABAergic system. Administration of allopreganolone to rats decreased vasopressin mRNA expression in the ventromedial subdivision of the hypothalamic PVN (Patchev *et al.* 1996). In the present study, removing the inhibitory effect of allopregnanolone through GABA receptors, by administration of 4-MA, increased the ACTH response in pregnant rats after swimming stress. It is proposed that this is the result of increased CRH and/or vasopressin secretion by the hypothalamus during stress which is normally enhanced without the inhibitory action of allopregnanolone.

Another 5α-reductase inhibitor, finasteride, does not change either cerebral cortex or plasma progesterone concentration, however it reduces significantly allopregnanolone and THDOC concentrations in the brain and plasma in day 20 (Concas *et al.* 1998; Concas *et al.* 1999) or in day 21 pregnant rats (Frye & Bayon 1998), from their high values during pregnancy, to close to the low values of estrus. The concentrations of these two neurosteroids in the cerebral cortex were measured by Concas et al. (Concas *et al.* 1998) after finasteride administration. Two hours after giving finasteride, neurosteroid concentrations in day 20 pregnant and estrus rats were reduced to similar levels (allopregnanolone: from 3.0±0.3 to 0.7±0.08 ng/g in estrus, from 12.2±1.6 to

1.2±0.1 ng/g in pregnant rats; THDOC: from 1.5±0.2 to 0.4±0.07 ng/g in estrus, from 2.8±0.4 to 0.3±0.09 ng/g in pregnant rats). Although finasteride decreases both neurosteroid concentrations in estrus, the actual values of these neurosteroids before finasteride administration were lower than in pregnancy.

An interesting result from the present study was the prolonged increase in ACTH secretion after stress in the 4-MA treated virgin and pregnant rats. This suggests that both neurosteroids, produced during stress, and $GABA_A$ receptors, are involved in the negative feedback that returns HPA axis activity to basal after the stressor is removed (Jessop 1999). Intraventricular injection of 10 µl anti-allopregnanolone serum significantly potentiates plasma corticosterone response to an acute cold water swimming stress (Guo *et al.* 1995). The administration of allopregnanolone and its precursor progesterone also resembles glucocorticoids in their suppression of the pituitary-adrenal response to emotional stress (Patchev *et al.* 1996). The prolonged ACTH responses to stress in the 4-MA treated rats provides indirect evidence for the effectiveness of 4-MA in inhibiting neurosteroid formation.

Allopregnanolone and $GABA_A$ receptors are localised in a broad distribution in the brain and exert many behaviour roles apart from the inhibitory action on the HPA axis system, such as anticonvulsant (Belelli *et al.* 1989), and anxiolytic effects, but effects of 4-MA on these aspects were not assessed in the present study.

4-MA and exogenous CRH-induced ACTH secretion

In the present study, the injection of CRH (200 ng/kg) 70 min after the swim, showed there was no statistically significant difference in ACTH increment within the virgin and pregnant groups (Figure 7.4), whether they were given vehicle or 4-MA. This suggests that the reduced allopregnanolone production induced by 4-MA administration may not have directly affected the corticotrophs. However, we cannot be certain about this due to the small number of samples, because in the pregnant 4-MA group the 3 rats that were able to be sampled may not have been representative (Figure 7.4). The tendency for a greater response to CRH in the pregnant 4-MA group is consistent with increased endogenous vasopressin secretion after 4-MA treatment (see Chapter 5). It is unlikely that allopregnanolone directly affects corticotrophs, as *in vitro* studies, the neurosteroids dehydroepiandrosterone, pregnenolone and

pregnanolone do not affect POMC mRNA expression in pituitary AtT 20 cells, even at the highest concentrations employed (Vedder *et al.* 1993). Although some studies reported that GABA has a stimulatory effect on ACTH secretion directly on corticotrophs (Anderson & Mitchell 1986; Tominaga *et al.* 1989), others found GABA has no direct effect on the anterior pituitary (Loeffler *et al.* 1986) and GABA receptors are not reported in the corticotrophs so far.

Immediately before parturition, progesterone, allopregnanolone and THDOC concentrations in plasma and brain are reduced to close to basal levels (Concas *et al.* 1999). This reduction of the concentrations of these hormones is believed to trigger parturition. In the present study, the artificial decrease in allopregnanolone and THDOC levels by 4-MA did not lead to signs of pre-term birth.

$GABA_A$ receptors have been identified in both the uterine myometrium and endometrium (Amenta *et al.* 1988). Allopregnanolone has a stronger effect than progesterone on inhibition of uterine contraction both *in vivo* (Majewska & Vaupel 1991; Putnam *et al.* 1991) and *in vitro* (Cabral *et al.* 1994; Thornton *et al.* 1999) through $GABA_A$ receptors. Allopregnanolone is considered to participate in regulation of $GABA_A$ receptors (Majewska *et al.* 1989) and their subunits (Fujii & Mellon 2001) in the uterus during pregnancy. In the present study, the changes (uterine blood vessels appeared more distended in the 4-MA treated pregnant rats) in the uterus may be due to the reduction of allopregnanolone production by 4-MA administration, however, the mechanisms of uterine blood vessels distended by 4-MA treatment is not clear.

5α-reductase also catabolises other hormones besides progesterone (review: (Celotti *et al.* 1992)): testosterone to 5α –androstan-17β-ol-3-one (5α-dihydrotestosterone) and androstenedione to 5α- androstan-3,17-dione (androstandione), so 4-MA would also block these conversions. However, there is little basis for considering interference with these conversions by 4-MA as a mechanism for reversing the attenuated HPA axis responses to stress in pregnancy. 5α-reductase also catalyses the conversion of corticosterone to 5α –pregnan-11β,21-diol-3,20-dione (dihydrocorticosterone) and also the conversion of deoxycorticosterone to 5α –pregnan-21-ol-3,20-dione

(dihydrodeoxycorticosterone). But the possible physiological role of the 5α-reduction of corticoids is unknown.

In conclusion, high brain and plasma concentrations of neurosteroids in late pregnancy are important for the attenuated ACTH response to stress. This action is through allopregnanolone inhibition of secretion (and possibly production) of CRH and vasopressin by parvocellular PVN neurones, rather than inhibition of ACTH secretion through direct action on corticotrophs in the anterior pituitary. Neurosteroids are involved in the switching-off of the HPA axis response to stress in virgin and pregnant rats.

Chapter 8

General discussion

In late pregnancy, ACTH secretion from anterior pituitary corticotrophs in response to stress is attenuated and the sensitivity of the adrenal cortex is increased. This attenuated ACTH secretion would provide a protective mechanism for the fetuses, as high levels of corticosterone in the plasma damage the development, growth, and future mental health of the pups. The mechanisms underlying the attenuated ACTH response many comprise two broad mechanisms: changes in corticotroph sensitivity and in secretagogue exposure.

The changes underlying the attenuated ACTH secretion from corticotrophs during pregnancy are summarised in Figure 8.1.

8.1 changes in gene expression of controlling ACTH secretion factors in the anterior pituitary

The density of the two major ACTH secretagogue receptors, the CRHR1 and V1b receptors, on corticotrophs are reduced in pregnancy as shown by receptor binding studies (Neumann *et al.* 1998; Toufexis *et al.* 1999). In the present study, we found no change in CRHR1 and reduced V1b receptor mRNA expression during pregnancy. Glucocorticoid negative feedback mediated by GR was likely to be enhanced as suggested from the increased GR mRNA expression in the anterior pituitary. This enhanced mechanism was also supported by the effect of phADX on POMC mRNA expression: phADX caused a greater reduction of corticosterone concentration in pregnant rats (by 32 ng/ml and 85 ng/ml in virgin and pregnant rats respectively), but the increases in POMC mRNA expression were about the same in virgin and pregnant rats after phADX.

Figure 8.1 Summary of the changes in the HPA axis during pregnancy.

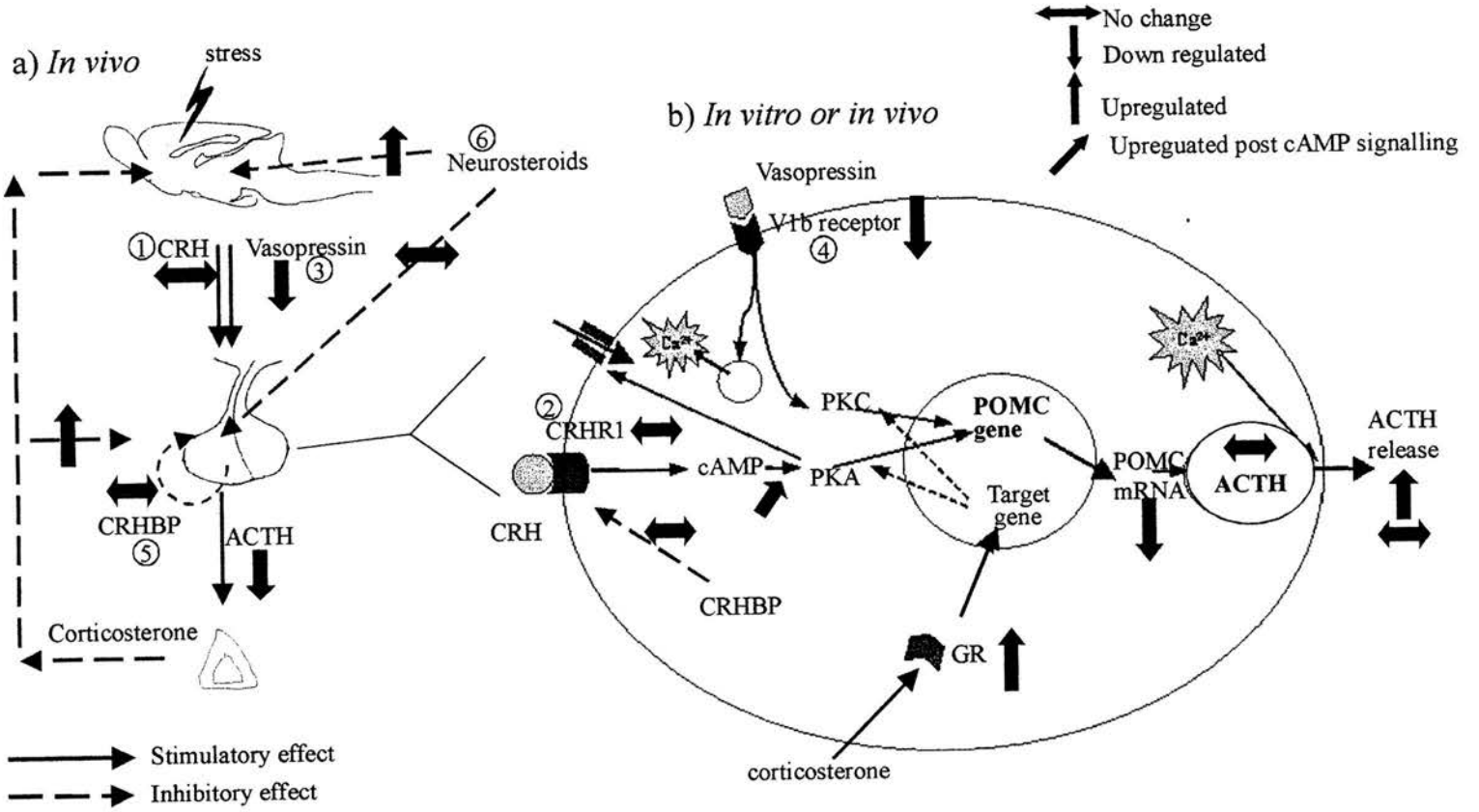


Figure 8.1 Summary of the changes in the HPA axis during pregnancy.

a) Summary of changes in the effects of stress-induced endogenous CRH and vasopressin on ACTH release. During pregnancy, in response to stress, compared to virgins, the effect of CRH was not changed; the effect of vasopressin was reduced; the inhibitory effect of neurosteroids was enhanced in the brain, but not in the corticotrophs; the effect of glucocortcoid feedback was enhanced. All of these changes contribute to attenuated ACTH response to stress.

b) Summary of changes in corticotroph responses to CRH and vasopressin *in vivo* and *in vitro*. In pregnancy, CRHR1 mRNA expression did not change, but cAMP signalling was enhanced; V1b receptor mRNA expression was decreased; GR mRNA expression was increased; CRHBP mRNA expression was not changed; POMC mRNA expression was reduced. All of these changes together, contribute to enhanced ACTH release *in vitro* or no change *in vivo* in response to CRH and vasopressin in combination.

② ④ ⑤ details in Chapter 3;. ① details in Chapter 4; ③ details in Chapter 5; ⑥ details in Chapter 7.

Expression of the mRNA for CRHBP, another factor indirectly inhibiting ACTH secretion, and its stimulation by stress, were similar in pregnant rats and in virgin rats, suggesting that this protein may not be important in regulating the effectiveness of CRH for ACTH secretion during pregnancy. No change of BK channel and STREX mRNA expression suggests no change in the role of these channels in regulating ACTH secretion in pregnancy. The changes in mRNA expression in the anterior pituitary that were found may contribute to reduced ACTH secretion, or be secondary to changes in the hypothalamic secretagogue input. To further investigate the importance of possible change in the exposure of corticotrophs to the secretagogues, we measured the ACTH response to CRH and vasopressin *in vivo* (Chapter 4 and Chapter 5), and the effects of CRH and vasopressin receptor antagonists on stress responses.

8.2 CRH and vasopressin on ACTH secretion *in vivo* and *in vitro*

CRH and vasopressin act synergistically to stimulate ACTH secretion. Thus the attenuated ACTH response to exogenous CRH and vasopressin in pregnancy indicates either decreased corticotroph sensitivity, or reduced basal CRH and vasopressin release in pregnancy. However, the combination of CRH plus vasopressin stimulated ACTH secretion similarly in virgin and pregnant rats, suggesting the ability of the corticotrophs to secrete ACTH is not different between virgin and pregnant rats and that the reduced ACTH response to CRH or vasopressin alone is due to lower basal levels of endogenous CRH and vasopressin in pregnancy.

Blocking CRH action using the CRHR1 antagonist, antalarmin, reduced the ACTH response to swim stress similarly in virgin and pregnant rats indicating that stress-induced CRH release may not be changed in pregnancy. However, CRH available for stimulating ACTH secretion in the median eminence was lower in pregnancy, although this may reflect reduced CRH production related to reduced daily secretion under basal conditions in the pregnant rats. The V1a/b receptor antagonist reduced the ACTH response to swim stress only in virgin rats, but not in pregnancy, which

suggests that stimulated vasopressin secretion is decreased in pregnancy and this may be the important reason for the attenuated ACTH response to stress in pregnancy.

In contrast to the results from the *in vivo* studies, ACTH secretion by corticotrophs *in vitro* was greater in pregnancy in response to CRH. This is probably due to enhanced cAMP post receptor signalling in corticotrophs, despite the receptors for CRH and vasopressin on the corticotrophs being reduced in pregnancy. Consistent with the *in vivo* result, the effect of combination of CRH plus vasopressin on ACTH secretion *in vitro* was not reduced in pregnancy. In pregnancy, CRHR1 density in the anterior pituitary is reduced, however, the signalling through cAMP is increased, this increased efficiency may be important to maintain an ACTH response.

8.3 A role for neurosteroid in the attenuated ACTH response in pregnancy

Progesterone and its neurosteroid metabolite levels are increased progressively during pregnancy, reaching peak values around day 20, then returning to basal just before parturition. Inhibitory effects of progesterone and allopreganolone on the HPA axis are already known. In the present study, blocking production of neurosteroids from progesterone by 4-MA enhanced the ACTH responses to swim stress in pregnant rats, but not to exogenous CRH. The enhanced inhibitory effect exerted by progesterone metabolites in pregnancy may be the reason for the attenuated ACTH response to stress in pregnancy. As exogenous CRH- induced ACTH secretion was not changed by administration of 4-MA, the neurosteroid metabolites may act on the hypothalamus to reduce CRH and/or vasopressin synthesis and release, rather than inhibit ACTH secretion directly from the corticotrophs, although the sample size for the interaction of 4-MA and exogenous CRH on ACTH secretion was small.

It was evident that in both virgin and pregnant 4-MA treated rats the increase in ACTH secretion was more prolonged after stress than in the vehicle-treated rats. This may reflect a role for inhibitory neurosteroids in terminating the stress response. Inhibitory neurosteroids are expected to act by interaction with $GABA_A$ receptors,

which mediate inhibitory actions of GABA pathways on CRH neurones. This requires further investigation.

8.4 Physiological importance

The HPA axis and the female reproductive system exhibit a complex relationship, the HPA axis exerting profound, mostly inhibitory effects, on the reproductive axis, with CRH and CRH-induced POMC peptides inhibiting hypothalamic GnRH secretion, and glucocorticoids inhibiting pituitary LH and ovarian estrogen and progesterone secretion, and suppressing the gonadal axis at the levels of the hypothalamus, pituitary, gonads, and end-target tissues (Rivest & Rivier 1995).

Pregnancy is associated with an increased incidence of mental illness and mood disturbance in both the immediate antenatal and post-natal period. Plasma levels of CRH, from the placenta, are markedly elevated in pre-eclamptic mothers (Perkins *et al.* 1995), and in healthy pregnant women during normal labour. Placental CRH is proposed to be the biological clock that times labour and delivery in women from the evidence that women who deliver prematurely have higher levels of CRH, about the same values of those of women close to term; and lower levels of CRH in women with post-mature delivery (McLean *et al.* 1995). The postpartum period is characterised by an increased incidence of psychiatric problems: "postpartum blues", a mild form of transient depression (Magiakou *et al.* 1996). This may be linked to altered HPA axis regulation, perhaps involving action of placental CRH.

Marked changes in concentrations of circulating hormones are observed during pregnancy. Much interest has focused on the potential changes in the HPA axis during pregnancy and the development of maternal postnatal depression and affective disorders. However, the mechanisms of these changes are not clear. The finding of decreased CRH gene expression in the PVN during pregnancy in the rats suggests that reduced hypothalamic CRH secretion occurs during pregnancy (Johnstone *et al.* 2000). In humans, secretion of CRH from the placenta into the circulation has been postulated to influence the maternal HPA axis (Waddell 1993), however,

measurements of the plasma levels of CRH during pregnancy do not correlate with the development of mood disorder (Smith *et al.* 1990). It is interesting to note that the abnormalities in HPA axis activity continue for several weeks after delivery when the plasma levels of CRH have returned to pre-pregnancy levels (Owens *et al.* 1987) and altered HPA axis activity occurs in pregnancy in species such as rodents (Atkinson & Waddell 1995) which do not possess elevated levels of plasma CRH during pregnancy. The development of postnatal mood disturbances was found to be significantly related to plasma cortisol levels measured at 38 week of pregnancy (Handley *et al.* 1980).

It is also clear that exposure of fetuses to stress, and particularly to glucocorticoids has lifelong adverse effect (Welberg & Seckl 2001), a "programming" with persistent organisational effects including hyperactive HPA system, increased anxiety and reduced ability in cognition. Maternal stress during week 28-30 of gestation is associated with birth outcome: increased levels of psychosocial stress are significantly related to gestational age at birth and infant birth weight. Maternal stress during the third trimester was associated with increased maternal plasma levels of ACTH and cortisol (Sandman *et al.* 1997). Prenatal maternal stress has been related to neonatal activity and irritability. Animal research demonstrates that prenatal stress feminises and demasculinises the sexual behaviour of males and reduces fertility and fecundity in females, producing estrous cycle disorders, spontaneous abortions, or vaginal hemorrhaging and high neonatal mortality (Herrenkohl 1986).

The neuroendocrine response of the HPA axis is required for the survival of the organism during times of stress, Therefore progressive a reduced stress response would be expected to be harmful to the mother. However, pregnant rats survive acute experimental stress well (Neumann *et al.* 1998). We deduced that vasopressin secreted from the hypothalamus into the portal blood during stress is reduced, and there were secondary adaptive changes in the signalling system of the corticotrophs in pregnancy of rats, suggesting rats may protect themselves and their offspring by these mechanisms.

8.5 Future studies

To further elucidate the mechanisms underlying the attenuated ACTH secretion from corticotrophs during pregnancy, the following information would be valuable. 1) direct measurement of hypothalamic changes in vasopressin release; 2) measurement of vasopressin or CRH action on ACTH secretion alone *in vivo*; 3) details of changes in post receptor signalling in corticotrophs; 4) roles of neurosteroids in the brain.

1) Knowledge of changes in the median eminence vasopressin content in pregnancy, including after stress, will be important for understanding the attenuated ACTH response to stress. Although it is difficult to measure vasopressin of PVN parvocellular neurone origin in the median eminence directly, as the median eminence contains vasopressin from both the parvocellular (external zone) and magnocellular (internal zone) neurones, vasopressin levels in the median eminence can be evaluated by immunohistochemistry.

2) In the present study, we used the V1a/b receptor antagonist to investigate the role of vasopressin and CRH in ACTH secretion. This antagonist blocked the effects of vasopressin via V1b receptors as well as V1a receptors, so in order to elucidate the exact action of vasopressin on the corticotrophs through the V1b receptors in pregnancy, a more specific V1b receptor antagonist (or a V1a receptor antagonist) would be useful. The CRHR1 antagonist used in the present study, antalarmin, is a non-peptide compound which can pass the blood-brain barrier and have central actions via the CRHR1 in the brain, so to elucidate CRH action on the corticotrophs use of a peptide antagonist would be valuable. To further confirm importance of the reduced basal CRH and stress-stimulated vasopressin release and to remove the interaction of vasopressin and CRH respectively, treatment with a specific V1b antagonist and then exogenous CRH, and a peptide CRHR1 antagonist and then exogenous vasopressin, and measurement of ACTH secretion in pregnancy would be appropriate.

3) CRH post-receptor signalling is enhanced in pregnancy as suggested by the incubation of acutely isolated anterior pituitary with 8-CPT-cAMP. This increased effect of cAMP may be via calcium influx through L-type calcium channels, so it

would be interesting to know the changes in intracellular calcium concentrations in response to CRH or cAMP, and the calcium channel function and electrophysiological properties in pregnancy.

4) 4-MA is a 5α-reductase inhibitor preventing the metabolism of progesterone to its metabolites and has been reported to affect behaviour by blocking the production of progesterone neurosteroid metabolites. However, in the present study, we did not measure the plasma and brain concentrations of progesterone metabolites, allopregnanolone and THDOC. Measurement of changes in these neurosteroid concentrations would offer more evidence of the role of the neurosteroids on the ACTH response to stress in pregnancy. The 5α-reductase inhibitor, 4-MA, prevents not only the production of allopregnanolone and THDOC, but also blocks production of other metabolites of steroid hormones such as dihydrotestosterone from testosterone, androstandione from androstenedione, and dihydrocorticosterone from corticosterone. To further investigate the role of allopregnanolone in the ACTH response, the administration of allopregnanolone after 4-MA would be a useful option. To investigate the sites of the neurosteroid action, after 4-MA or allopregnanolone administration, CRH and vasopressin gene expression in the PVN, and the ACTH response to exogenous CRH also should be measured.

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(10^{-6} M), and mimicked by an agonist, 4-Thr-7-Gly-OT (10^{-7} M). Analysis of immunoidentified profiles showed that as in vivo, the changes were specific to OT neurons. Finally, the unbiased disector method revealed that OT application induced an over 30% increase in the overall synaptic density of the SON.

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P2-3

Generation of burst activity in supraoptic oxytocin neurones in vivo: role of dendro-dendritic interaction and intracellular Ca2+.

Leng G., Ludwig M. & Sabatier N.
Div. Biom. Clin. Lab., Edinburgh, uk

Dendritic release of peptides from supraoptic nucleus neurones is thought to play an important role in the regulation of these neurones. Oxytocin (OT) has been shown to have feed-back action on OT neurones: it facilitates the generation of milk-ejection bursts in rats in vivo, induces its own dendritic release both in vitro and in vivo and triggers an increase in intracellular Ca2+ concentration ([Ca2+]i) through mobilisation of internal Ca2+ stores. Interestingly, variations in [Ca2+]i seem to be a key factor in determining the firing pattern, either continuous or phasic, of supraoptic neurones. In this study, we combined [Ca2+]i increase and dendritic release within the supraoptic nucleus to investigate the effect of these factors on firing pattern in OT neurones. Thapsigargin, an intracellular Ca2+ mobiliser, was applied through a microdialysis probe placed on the exposed ventral surface of the supraoptic nucleus in adult virgin female rats. In combination with thapsigargin administration, we triggered dendritic release by using the technique of constant collision stimulation. Extracellular single cell recordings were performed in OT neurones. We found that the electrical activity of OT neurones switched from a continuous pattern to short clusters of action potentials (5 to 7 spikes long) and in some cases, milk ejection-like bursts were observed. Fine analysis showed that the relationship between each interspike interval and the interval that precedes turns into a positive correlation, indicating that short intervals are more likely followed by short intervals. The correlation is lost when intervals are randomised and negative correlation appears when a number of intervals exceeding the cluster length is analysed. In conclusion, our results suggest that local feed-back mechanisms involving variations of intracellular Ca2+ concentration generate a switch in the activity of OT neurones into a pattern more favorable for the appearance of milk ejection bursts.

P2-4

Vasopressin and the attenuated ACTH responses to stressors during pregnancy in rats.

Ma S. (1), Shipston M. (2) & Russell J. (1)
(1) Lab Neuroendocrinology, Edinburgh, UK ; (2) MBG, Edinburgh, UK

Vasopressin (VP) is co-produced by parvocellular paraventricular nucleus (pPVN) corticotropin releasing hormone (CRH) neurones. Alone, VP weakly stimulates ACTH secretion in rats, but it augments the action of CRH. ACTH secretory responses to stressors are attenuated in late pregnant rats, and we have investigated whether altered release or action of VP is involved. To test in vivo VP sensitivity, ACTH was measured in jugular venous blood before and after bolus i.v. VP injection (1.7 µg/kg) : ACTH secretion increased less in pregnant (day 21) than in virgin rats (by 44 % at 5 min ; $p<0.05$, two-way ANOVA). A V1a/b antagonist (dP(Tyr(Me) 2, Arg-NH29]AVP, 10 mg/kg, i.v., shown to prevent VP stimulation of ACTH secretion ; a gift from M. Manning), or vehicle was given before swim stress (90 sec) : after vehicle, ACTH secretion was increased less by swim-

ming in pregnant than in virgin rats (by 200 pg/ml vs 483 pg/ml at 5 min) ; the V1a/b antagonist reduced the increase in ACTH secretion more in virgin than in pregnant rats. Using a V1b receptor mRNA riboprobe (a gift from S. Lolait), for quantitative in situ hybridisation (ISH) on pituitary cryostat sections, we found reduced expression in pregnancy (by 19 % vs. virgins). To test in vitro sensitivity to VP, pituitaries were removed from virgin and pregnant rats and cell suspensions prepared ; increasing concentrations of CRH and VP, alone or together, were added to incubated aliquots for 60 min, and ACTH release measured. Stimulation of ACTH secretion by CRH was similar for cells from pregnant and virgin rats, but augmentation of CRH effects by VP was greater in pregnancy. This may be a result of enhanced post-receptor mechanisms, as a membrane permeable cAMP analogue more effectively stimulated ACTH secretion by pituitary cells from pregnant rats. Overall, although corticotrophs are more responsive to VP augmenting actions, this does not compensate for probable reduced CRH secretion in response to stress.

P2-5

The effects of the afterhyperpolarisation on firing pattern in vivo: a statistical approach.

Marganiec A. (1), Moos F. (2) & Leng G. (1)
(1) Biomed Sci, Edinburgh, UK ; (2) CNRS UMR5101, Montpellier, France

In vitro studies in magnocellular neurosecretory cells (MNC's) of the supraoptic nuclei (SON) showed that trains of high frequency discharge (20-50 Hz) are followed by an afterhyperpolarisation (AHP), which lasts around 1s and is generated by the activation of a calcium dependent potassium channel. It has been suggested that the AHP is of importance in the regulation of continuous and phasic firing in SON cells, and its inactivation may be necessary to enable MNC's to fire at the high frequencies that occur during milk-ejection bursts. However, since in vitro experiments include the injection of a depolarising current and hence create an artificial situation the physiological role of the AHP in vivo remained unclear. To investigate whether the AHP has a role in the in vivo activity of oxytocin neurones, the present study employed statistical methods to analyse in vivo extracellular recordings of virgin and lactating rat.
We investigated the relationship between successive interspike intervals (ISI's) of long recordings of continuous firing cells and the background activity of cell displaying high frequency discharges associated with milk-ejection burst.
We report a non-random distribution of spikes, whereby a short interval is more likely to be followed by a long interval, and vice versa. If the AHP has an impact at spontaneous firing rates in vivo, we would expect to find a balancing effect, i. e. a period of slower activity should follow after a period of more intense activity. This hypothesis is consistent with our observations of in vivo firing patterns.

P2-6

Thymic neurohypophysial peptide-mediated signalling and T-cell differentiation.

Martens H., Hansenne I., Brilot F., Rasier G., Charlet-Renard C. H., Legros J. J. & Geenen V.
Inst. Pathol., CHU-B23, Liège, Belgium

Thymic epithelial cells (TEC) express a repertoire of neuroendocrine-related genes characterised by the dominance of one member over others. Recapitulating the dual role of the thymus in T-cell differentiation, this repertoire of genes codes either for cryptocrine ligands binding to cognate neuroendocrine receptors expressed by pre-T cells, or for neuroendocrine self-antigens presented by thymic MHC to rearranged TCR on thymocytes. In the neurohypo-

polysialylated NCAM (PSA-NCAM), F3/contactin and its ligand the matrix glycoprotein, tenascin-C. PSA-NCAM is of particular relevance since the complex carbohydrate PSA on its extracellular domain reduces cell adhesion, thereby allowing surface interactions underlying the dynamic cell changes responsible for such plasticity. Our recent ultrastructural analyses have provided direct evidence that PSA-NCAM is indeed a prerequisite since a single microinjection of endosialidase, an enzyme that selectively cleaves PSA from NCAM, close to the supraoptic nucleus in vivo, inhibited the morphological changes normally occuring in response to lactation (J Neurosci, 1999, 19, 10228). On the other hand, our in vivo and in vitro observations demonstrate that OT itself, in synergy with sexual steroids, can induce such plasticity since its application (by microinfusion into the 3rd ventricle or perifusion of acute hypothalamic slices) leads to neuronal-glial and synaptic changes similar to those brought on by parturition and lactation.

S132

Protective brain adaptations of stress coping mechanisms in pregnancy and lactation: involvement of oxytocin and prolactin

I. D. Neumann (Munich, D)

Pregnancy and lactation are accompanied by a reduced responsiveness of the hypothalamo-pituitary-adrenal (HPA) axis and the oxytocinergic system to a variety of stressors. Studies on rats have shown that these changes partly triggered by alterations in circulating steroidal hormones are due to a combination of attenuated stressor perception and feedforward mechanisms at limbic, hypothalamic and anterior pituitary levels involving also the CRH system. Further, maternity is accompanied by dramatic alterations in the emotionality with an increased level of anxiety-related behaviour in late pregnant rats. In lactation, anxiety is reduced which is possibly a prerequisite for increased (maternal) aggression and defence of the offspring. The robust neuroendocrine and emotional alterations seen in pregnancy and lactation are independent of the inborn level of anxiety as shown in rats bred for either high or low anxiety-related behaviour.

Various neuropeptides highly activated at this time to meet reproduction-related demands like oxytocin, prolactin and endogenous opioids were shown to significantly regulate the (re)activity of the HPA axis and/or to exert anxiolytic effects. Such neuropeptides are, therefore, possible candidates to be involved in the attenuated neuroendocrine stress responsiveness and altered emotionality peripartum. These physiological and reversible adaptations are, in pregnancy, likely to contribute to the protection of the fetuses from exposure to excessive, and damaging, levels of glucocorticoids. In lactation, they may protect the mother from catabolic processes caused by glucocorticoids. Further, down-regulation of the activity of the brain CRH system may be a protective mechanisms to cope with the dramatic hormonal fluctuations in this period. Disturbance of these mechanisms, e.g. by repeated exposure to stress throughout pregnancy, results in an enhanced neuroendocrine stress responsiveness and increased anxiety in lactation.

Supported by DFG (Ne 465).

S133

Lactation-induced changes in amygdala-paraventricular nucleus (PVN) connections: are they mediating emotional filtering?

C.-D. Walker, S. Deschamps, A. Burlet (Verdun, CDN; Nancy, F)

Lactation is associated with multiple physiological and behavioral changes that are optimal for development of the offspring. In particular, neuroendocrine stress responses and some emotional responses are blunted, but it is still unclear whether this phenomenon is dependent upon the nature of the stressor and whether specific alterations in neuronal circuitry allow to "filter" stressful stimuli during lactation. Corticotropin releasing factor (CRF)-containing neurons in the hypothalamic PVN control neurodendocrine stress responses and are modulated by inputs from the brain stem, the amygdala, and the bed nucleus of the stria terminalis (BNST) which integrate emotions. Here, we tested the hypothesis that 1) lactation induces changes in the PVN-BNST-amygdala relationships that are directed towards maintaining low reactivity to infant-irrelevant stimuli and 2) stressors that threaten survival of the infant can overcome this "filtering" mechanism and activate important neuroendocrine and behavioral responses in the mother. Compared to virgins, lactating rats exhibit higher CRF mRNA expression in neurons of the dorsolateral BNST, which relay some of the inhibitory hippocampal inputs to the PVN and reduced CRF mRNA levels in the central amygdala (CeA), which is considered stimulatory to PVN neurons. Using CRF-directed immunolesions of the PVN or PVN+BNST neurons, we showed that in lactating females, PVN neurons containing CRF maintain a tonic inhibition over neurons in the anterior BNST and the CeA. This further suggests that reciprocal connections between the PVN and the BNST/amygdala in lactating females are modified to lower the influence of stimulatory inputs on stress-responsive PVN neurons. However, this "filtering" system can be superseeded since nursing mothers exhibit important neuroendocrine responses to a stimulus causing a direct threat to their pups, i.e. a male intruder, compared to a physical stressor less relevant to their litter. We are currently testing whether additional brain structures are recruited after specific stressors, that might overcome the hyporesponsiveness of the PVN-BNST/amygdala circuitry during lactation.

S134

Pregnancy reduces neuroendocrine stress responses: attenuated central processing of stressors and activation of opioid inhibition

J. A. Russell, P. J. Brunton, H. A. Johnstone, S. Ma, J. R. Seckl, I. D. Neumann, A. J. Douglas (Edinburgh, UK; Munich, D)

Brain pathways processing different stressors converge onto the corticotropin-releasing hormone (CRH)/vasopressin (VP) neurones in the parvocellular paraventricular nucleus (pPVN) of the hypothalamus, which stimulate ACTH secretion from the anterior pituitary and hence glucocorticoid secretion from the adrenal cortex. Simultaneously, centrally-projecting CRH neurones are activated, eliciting anxiety-related behaviours. We have investigated changes in the brain mechanisms regulating the neuroendocrine stress response in pregnancy. In late pregnant rats, basal activity of the pPVN neurones is decreased in terms of CRH and VP gene expression (measured by in situ hybridisation), and the diurnal rhythm in ACTH secretion is suppressed. Furthermore, pPVN neurone mRNA responses (for the immediate early gene NGF-IB, and the CRH and VP genes) to stress are attenuated in late pregnancy. These attenuated responses underlie reduced ACTH secretory responses to emotional and mild physical stressors, including immune challenge. These changes may result from reduced secretory capacity of the CRH/VP neurones, while the pituitary ACTH content is normal. Glucocorticoid negative feedback is evidently enhanced as 11beta-hydroxysteroid dehydrogenase activity (reactivating corticosterone) in the PVN and glucocorticoid receptor mRNA expression in the dentate gyrus are increased. Consequently, decreased activity or effectiveness of excitatory inputs to CRH/VP neurones is likely. This is supported by finding

lack of stimulation of NGFI-B mRNA expression in the ippocampus by stress in pregnancy.

Activation of central endogenous opioid inhibitory mecha-isms, important in hypoalgesia and in restraining stress activa-on of oxytocin neurones in pregnancy, is also involved. Hence ie opioid antagonist naloxone restores ACTH responses to ress or systemic interleukin-1beta. The reduced neuroendocrine ress responses in pregnancy may protect the fetuses from dverse programming by excess glucocorticoid, but the underly-g changes in stressor processing and reduced stress activation in ie brain have implications for maternal mood changes post artum.

Support: BBSRC; DAAD/British Council.

135

he neuroendocrine basis of social attachment

. S. Carter (College Park, MD, USA)

he purpose of this presentation is to review the neuroendocrine echanisms responsible for both the formation of social bonds nd those mechanisms through which social bonds influence ental health. Social attachments and pair bonds facilitate both production and survival, providing a sense of safety and ducing anxiety or stress. Studies of social attachment have cused on mother-infant and adult pair bonds. The underlying bstrates of both may rely on shared neuroendocrine substrates. t the core of the neurobiology of social bonds is an ancient stem which utilizes several neuropeptides including oxytocin. here also is a recurrent association between exposure to stressful periences [and hormones of the hypothalamic-pituitary-adrenal HPA) axis] and the subsequent formation of social bonds. ositive social experiences, including social bonds, may reduce PA axis activity. In addition, oxytocin is capable both of cilitating the formation of social attachments and inhibiting PA and autonomic reactivity. Of particular interest is the fact at these systems are designed to be modified by social experi-ces in early life, which can in turn produce long-lasting changes the neuroendocrinology of social behavior. For example, early posure to oxytocin or the related peptide, vasopressin can rmanently modify both behavior and central peptide receptors. n understanding of the biological basis of social attachment and developmental consequences provides insights into the euroendocrine mechanisms through which social support nefits mental and physical health. Failures of this system may lp to explain a variety of emotional disorders, including those at are characterized by deficiencies in social behavior.

ender differences in the genetics of mental sorders

136

ender differences in attitudes towards predictive testing d ethics

. Rietschel, F. Illes, G. Rudinger, M. Angermeyer, W. Maier onn, Leipzig, D)

ie advances in molecular genetics are producing insights into the olecular basis of a growing number of diseases. This knowledge ows to diagnose single-gene disorders as well as to identify edispositions to complex inherited disorders. Such testing may fer medical or psychological benefits but may also have adverse de-effects. The knowledge of being a carrier of a disease/ lnerability gene can help to treat/prevent the disorder, fur-ermore it may have an influence on family-planning, planning of e future, work, relationships, concept of an individual's autonomy and in general on his well-being. Testing can be performed in individuals at risk for a specific disorder or as screening of the general population.

Attitudes towards the potential benefits and negative consequences of this "new genetics" vary considerably, they are influenced by personal experiences, ethnical, religious, and political factors.

Women approach ethical dilemmas differently from men. In psychological studies of moral decision making women tended to use a relationships-based ethics that see individuals as embedded in a network of relationships while men were more likely to regard individuals as isolated agents.

The availability of presymptomatic testing for many different disorders will have an enormous impact on society in general and specifically on the relationships of parents/couples. They will have the option to decide whether to perform prenatal testing, predictive testing in their children or for themselves for a variety of possible disorders. Before the introduction of wide-spread DNA testing it would therefore be desirable to learn what femals and males think about this issue and if there are gender specific differences.

Within the framework of the German Human Genome Project we are conducting a representative survey on attitudes toward genetic research, counseling, potential misuse, and predictive testing in severe mental disorders. First results will be available by January 2001.

S137

Gender differences in the genetics of substance abuse

P. Franke (Bonn, D)

The etiology of substance abuse is presumed to be multifactorial. Within the last decade, family- (Merikangas et al. 1998), adoption- (Cadoret et al. 1995) and twin studies (Kendler et al. 2000) have underscored -apart from environmental and individual factors – the substantial role of genes in the development and maintenance of addictive behavior (Enoch & Goldman, 1999). The aim of psychiatric molecular genetics is to identify these genes leading to substance abuse and dependence.

From an epidemiological point of view prevalence rates of substance abuse disorders revealed gender differences which may reflect genetic differences between men and women. However, only few information is yet available on sex-specific genetic influences on substance abuse (which is often due to inadequate power to test for sex-specific transmission).

The following review will focus on this topic based on results of family-, adoption-, and twin study data as well as molecular genetic studies.

Cadoret RJ, Yates WR, Troughton E, Woodworth G, Stewart MA (1995) Adoption study demonstrating two genetic pathways to drug abuse. Arch Gen Psych 52: 42–52.

Enoch MA, Goldman D (1999) Genetics of alcoholism and substance abuse. Addictive Disorders 22: 289–299.

Kendler KS, Karkowski LM, Neale MC, Prescott CA (2000) Illicit psychoactive substance use, heavy use, abuse, and dependence in a US population-based sample of male twins. Arch Gen Psych 57: 261–269.

Merikangas KR, Stolar M, Stevens DE, Goulet J, Preisig MA, Fenton B, et al. (1998) Familial transmission of substance use disorders. Arch Gen Psychiatry 55: 973–979.

S138

Genetics of puerperal psychosis

I. Jones (Birmingham, UK)

For bipolar women the postnatal period is a time of heightened risk with episodes of severe psychiatric disturbance (puerperal

30.13

HYPTHALAMO-PITUITARY-ADRENAL AXIS STRESS RESPONSES IN PREGNANCY AND GLUCOCORTICOID NEGATIVE FEEDBACK

P.J. Brunton, S. Anderson, J. Bell & J.A. Russell
Dept. of Biomedical Sciences, University of Edinburgh, HRB, George Square, Edinburgh, EH8 9XD, UK.

We tested a role for enhanced glucocorticoid negative feedback in the hyporesponsiveness of the HPA axis to stressors in late pregnancy. In Expt. 1, to block corticosterone synthesis and feedback, virgin and pregnant rats were given metyrapone 8 hourly for 48h, and killed at 50h. Radioimmunoassayed plasma ACTH was increased, and corticosterone decreased. Cryostat sections of the parvocellular paraventricular nucleus (pPVN) were hybridised with 35S-oligo-probes complementary to vasopressin (VP) and CRH mRNAs. Film autoradiograph quantification showed increased VP and CRH mRNA expression in virgin and pregnant metyrapone-treated vs. vehicle-treated rats; expression was greater in the pregnant metyrapone group. In Expt. 2, to test rapid corticosterone feedback, virgin and pregnant rats fitted with a jugular vein cannula were blood sampled during forced swimming (FS; 90s at 19C) with either corticosterone or vehicle pretreatment. FS increased plasma ACTH in both vehicle-treated groups, but pregnant rats showed attenuation. Corticosterone treatment decreased the ACTH response to FS in virgin but not pregnant rats. Thus basal pPVN CRH and AVP mRNA expression is more sensitive to glucocorticoid feedback in pregnancy, but enhanced rapid feedback by corticosterone does not underlie HPA axis stress hyporesponsiveness in pregnancy. [Support: Medical Faculty Studentship (PJB), BBSRC].

30.14

CORTICOTROPH CHANGES REDUCING RESPONSIVENESS DURING PREGNANCY

Ma S, Douglas AJ, Shipston MJ & Russell JA
Dept of Biomedical Sciences, University of Edinburgh, EH8 9XD.

Anterior pituitary ACTH secretion is regulated by glucocorticoid feedback, corticotrophin releasing hormone (CRH) and vasopressin (VP). ACTH secretory responses to stressors and to CRH are attenuated in pregnant rats. Here, we quantified receptor (R) mRNAs for CRH1, VP1b and glucocorticoid (GR) in the anterior pituitary using in situ hybridisation (ISH). Comparing expression in pituitaries from virgin (n=9) and pregnant (day 21, n=6) rats: CRHR1 mRNA was not different; VP1b-R mRNA was reduced and GR mRNA increased in pregnancy (both $p<0.05$, t-test), the latter indicating increased negative feedback. To test in vivo VP sensitivity, ACTH was measured in jugular venous blood before and after i.v. VP bolus injection (1.7ug/kg): ACTH secretion increased less in pregnant than in virgin rats ($p<0.05$, two-way ANOVA). As CRF and VP synergistically elevate cAMP in corticotrophs we examined whether cAMP-stimulated ACTH release was modified in pregnancy using dispersed rat anterior pituitary cells. The cell permeable cAMP analogue (8-CPT-cAMP, 0.5mmole/l) stimulated ACTH release to a greater extent from cells isolated from pregnant rats than those from virgins. This enhanced efficacy of cAMP-stimulated ACTH release may compensate partly for the reduced VP1b-R expression in pregnancy. Support: ORS award (SM); Wellcome Trust.

30.15

SEASONALLY-INAPPROPRIATE BODY WEIGHT: EFFECT ON HYPOTHALAMIC GENE EXPRESSION IN SIBERIAN HAMSTERS

Mercer JG, Moar KM, Logie TJ, Findlay PA, Adam CL, Morgan PJ.
Aberdeen Centre for Energy Regulation and Obesity (ACERO), Molecular Neuroendocrinology Group, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, Scotland, UK

It is unknown whether the hypothalamus differentiates between weight change induced by imposed negative energy balance ("inappropriate" body weight) and seasonal weight change ("appropriate" body weight) in Siberian hamsters. Seasonally-inappropriate weight loss was induced in adult males (1) by food restriction in long days (LD) to mimic short day (SD) weight loss or (2) by food restriction in SD, superimposed on SD weight loss. SD weight loss was accompanied by reduced POMC but elevated CART mRNA in the arcuate nucleus (ARC) measured by in situ hybridisation. SD hamsters had reduced leptin receptor (OB-Rb) mRNA in the ARC and ventromedial nucleus (VMN); MC3-R mRNA was reduced in the ARC, but elevated in the VMN. Equivalent weight loss and low plasma leptin during LD-restriction produced mRNA profiles typical of negative energy balance, with low CART and elevated OB-Rb mRNAs in the ARC; MC3-R mRNA levels were indistinguishable from SD. Food restriction in SD up-regulated anabolic neuropeptide and OB-Rb mRNAs, indicating inappropriately low body weight despite ongoing SD weight loss. Thus, low plasma leptin is integrated differently in SD and LD-restricted animals. Many hypothalamic changes in SD are unlikely to be secondary to the seasonally-appropriate weight loss, but seasonally-inappropriate body weight in either photoperiod activates compensatory neuropeptide systems.

30.16

MODULATION OF NEURONAL ACTIVITY IN THE SUPRACHIASMATIC NUCLEUS BY AFFERENTS FROM THE RAPHE NUCLEUS

G.S. Bhumbra and R.E.J. Dyball
Department of Anatomy, University of Cambridge,Tennis Court Road, Cambridge, CB2 3DY.

Serotonin receptor agonists have been shown to shift the peak of single unit activity in the suprachiasmatic nucleus forwards or backwards, depending on the time of day they are applied. Electrophysiological experiments in vitro have shown that serotonin receptor agonists and antagonists are able to decrease and increase the excitability of cells in the suprachiasmatic nucleus. A serotonergic projection from the anterior group of raphe nuclei to the anterior hypothalamus has been described anatomically. The present study was designed to characterise the projection electrophysiologically in the rat brain. While electrically stimulating the region of the dorsal raphe, extracellular electrodes were used to record the single unit activity of cells in the suprachiasmatic nucleus in vivo. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Peristimulus-time histograms were constructed to demonstrate that most cells in the suprachiasmatic nucleus responded to dorsal raphe stimulation. Responses were either excitatory, or inhibitory, or both, with a range of latency from 0.04 seconds to 0.54 seconds. The results confirm that there is a functional input to the suprachiasmatic nucleus from the mesencephalon. The different nature of the responses suggests that the inputs are made up of a number of separate afferent pathways.

(P10) **ROLE OF CENTRAL VASOPRESSIN (VP) INFUSION ON RHYTHMS OF HYPOTHALAMO-PITUITARY-ADRENAL (HPA) ACTIVITY.** CD Ingram[1,2], MH Andrews[2], B Giddings[2], AJ Horsfall[1], YM Kershaw[2], CA Lowry[2], RJ Windle[3], SA Wood[2] and SL Lightman[2]. [1]School of Neurosciences and Psychiatry, University of Newcastle NE1 4LP, [2]University Research Centre for Neuroendocrinology, Bristol BS2 8HW, and [3]University of Nottingham, Lincoln County Hospital LN2 5QY

The HPA axis displays both ultradian (pulsatile) and circadian (diurnal) rhythms of secretory activity, with the amplitude of pulses being lowest at the time of lights off (nadir) and a peak at the time of lights on (acrophase). Central VP has been suggested to play a role in the control of these rhythms through its release from both the paraventricular nucleus and the suprachiasmatic nucleus (SCN), where the circadian clock resides. The following studies examined the effect of chronic (5 day) changes in VP levels on basal HPA activity. In the first study male Wistar rats were cannulated and administered with an i.c.v. infusion of either saline, VP or the V_{1a} antagonist [*d*$(CH_2)_5$,Tyr(Me)^2,Arg^8]VP via Alzet osmotic minipump for 5 days. Four days after surgery blood samples were collected every 10 min for 24 h and assayed for corticosterone (CORT). Rats infused with either saline or VP displayed regular pulses of CORT secretion the amplitude of which varied according to the predicted diurnal rhythm. In contrast infusion of the antagonist resulted in low amplitude pulses across the whole cycle, suggesting that blockade of VP neurotransmission disrupts the circadian input to the HPA. Ageing is associated with a gradual loss of VP-immunoreactive neurones in the SCN and we have previously reported that this correlates with a flattening of the circadian rhythm of HPA activity. To determine whether increasing endogenous VP levels would restore circadian activity, we tested the effect of continuous VP infusion i.c.v. on HPA activity in young (3-4 month) and aged (24 month) rats. Young rats displayed a clear diurnal pattern of CORT secretion with nadir levels of <20 ng/ml rising to peak levels of 70 ng/ml at the time of lights off. Infusions of VP had no effect on this pattern. Aged animals showed a predicted flattening of the rhythm, mainly due to an increase in nadir levels, and infusion of VP caused a restoration of the nadir levels similar to those in the young group. These data suggest that VP plays a major role in maintaining the diurnal rhythm of HPA activity, particularly through modulation of pulse amplitude in the early light phase period.

(P11) **ASSESSMENT OF CONTRIBUTIONS OF CRH AND VASOPRESSIN (VP) TO THE DRIVE TO CORTICOTROPES IN PREGNANT RATS.** Ma S, Douglas AJ, Shipston MJ & Russell JA; Dept of Biomedical Sciences, University of Edinburgh, EH8 9XD.

Stressors are less effective in stimulating ACTH and corticosterone secretion in pregnant rats than in virgins. Similarly, basal expression of CRH and VP mRNAs in the parvocellular paraventricular nucleus (pPVN) is reduced, as are responsiveness of corticotropes to CRH *in vivo*, and CRH receptor binding (Neumann et al 1998 J Physiol 501:289-301). We have further studied corticotrope responses to CRH and VP *in vitro* and *in vivo*. After decapitation, pituitaries were removed from virgin and day 21 pregnant rats and cell suspensions prepared; after incubation of aliquots for 2 h, increasing concentrations of CRH and VP, alone or together were added for 60 min, and ACTH released measured. Stimulation of ACTH secretion by CRH was similar for cells from pregnant and virgin rats, but augmentation of CRH effects by VP or stimulation by 8-cpt-cAMP, was greater in pregnancy. ACTH content per pituitary cell was similar between pregnant and virgin rats, but by quantitative *in situ* hybridisation on pituitary sections, POMC mRNA expression was significantly reduced at the end of pregnancy (n= 17; by 45% vs. virgins, n= 15). Evidently the corticotropes have a normal capacity to secrete ACTH in pregnancy. To test *in vivo* VP sensitivity, in the context of endogenous CRH/VP secretion, we measured ACTH concentration in jugular venous blood (cannula implanted under halothane anaesthesia) before and after i.v. VP bolus injection (1.7 μg/kg): ACTH secretion increased less in pregnant than in virgin rats. Thus, unlike in lactation (Toufexis *et al* 1999 J Neuroendocr 11:757-764), corticotrope sensitivity to VP is not increased in pregnancy. To study the contribution of CRH to ACTH stress responses, we gave a non-peptide CRH1 receptor antagonist, antalarmin (20 mg/kg, i.p., gifted by Drs Chrousos & Zoumakis, NIH), or vehicle, 90 min before swim stress (90 s): after vehicle, ACTH secretion was increased less by swimming in pregnant than in virgin rats, and antalarmin reduced the increase in ACTH secretion similarly in pregnant and virgin rats (by 52 and 53%, respectively; n= 10, 4). This suggests similar contributions by CRH to the ACTH response in pregnant and virgin rats. Together, the *in vitro* and *in vivo* data are consistent with reduced in vivo drive by both CRH and VP to the corticotropes in pregnancy; this is unlike in lactation, where VP is more, and CRH is less, important.

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184.15 *p210*

THE ROLE OF NEUROPEPTIDE Y IN THE ACTION OF OREXIN-A ON THE HYPOTHALAMO-PITUITARY-ADRENAL SYSTEM

M. Jaszberenyi, E. Bujdoso, G. Telegdy
Dept. of Pathophysiology, University of Szeged

The effects of the recently identified neuropeptide orexin-A on the hypothalamo-pituitary-adrenal (HPA) system, and the possible interactions between this peptide and neuropeptide Y (NPY) were investigated. An *in vivo* system was used to assess the central effects of orexin-A. Different doses of orexin (2.8-280 pmol) were administered intracerebroventricularly (icv.) to adult male Wistar rats, and plasma corticosterone was used as an index of the degree of the activation of the HPA system. Orexin-A exhibited a clear dose-response action, which showed a downturn phase, and pretreatment with the corticotropin-releasing hormone (CRH) antagonist α-helical CRH 9-41 completely prevented the action of orexin-A suggesting that the site of action is at a central level. As orexin-A is synthesized in the lateral hypothalamus and neuronal projections from this area to the paraventricular nucleus (PVN) have been identified, we examined whether NPY, a potent activator of the CRH secretion, participates in the action of orexin. In these experiments we injected different concentrations of NPY antiserum icv. to the rats 24 h prior to the administration of the most effective dose of orexin-A. The inhibitory action of the antiserum exhibited a dose-response curve, and the highest concentration completely abolished the action of orexin-A. This study affords strong evidence that this appetite regulating peptide activates the HPA system at a central level, and NPY appears to mediate the action of orexin. The animals were kept and handled during the experiments in accordance with the instructions of the Ethical Committee of University of Szeged for the Protection of Animals.

184.16 *p211*

TRH DEGRADING ECTOENZYME IN ADENOHYPOPHYSIS: CELLULAR LOCALIZATION, REGULATION BY TRH AND TRANSDUCTION PATHWAYS

J.L. Charli, M.A. Vargas, R. Cruz, L. Perez-Martinez, R.M. Uribe, P. Joseph-Bravo
Instituto de Biotecnologia, UNAM, Cuernavaca, Mexico

In the extracellular compartment, TRH is inactivated by a narrow specificity ectopeptidase, pyroglutamyl peptidase II (PPII). PPII is present in brain and in lactotrophs of adenohypophysis. We previously demonstrated that various hypothalamic-paracrine agents, including TRH, slowly (in hours) regulate the activity of PPII on the surface of adenohypophyseal cells in primary culture. The purpose of this study was to: 1) precise the cellular distribution of PPII in adenohypophysis, 2) identify the transduction pathway used by TRH to regulate PPII activity and 3) determine whether TRH regulates PPII activity *in vivo*. Using double in situ hybridization in primary cultures of female adenohypophyseal cells, we found that 3-6 % of cells express PPII mRNA; only half of these are of lactotrophs while none are thyrotrophs and the rest are yet unidentified cell types. In primary cultures of adenohypophysis, treatments enhancing intracellular calcium levels down-regulated PPII activity; their effect was not additive with the down-regulation induced by TRH. Regulation of PPII activity by TRH was inhibited by: a phospholipase C inhibitor, antagonists of L-type calcium channels, intracellular calcium chelators or, inhibitors of calmodulin or Cam kinase II. Inhibition of PKC or adenylate cyclase activities was not effective. In male rats, adenohypophyseal PPII activity and mRNA levels fluctuated during the circadian cycle. Intraperitoneal injection of TRH transiently reduced PPII activity. In conclusion, we have shown that PPII is expressed in a small sub-population of lactotrophs, that TRH down regulates PPII activity either *in vivo* or *in vitro* and that TRH-induced calcium entry mediates TRH effect on PPII activity. (Partially supported by grant 3299P-N9607 from CONACYT).

184.17 *p212*

CENTRAL MECHANISMS UNDERLYING REDUCED ACTH STRESS RESPONSES IN PREGNANT RATS: ATTENUATED ACUTE GENE ACTIVATION IN THE PARVOCELLULAR PARAVENTRICULAR NUCLEUS (PPVN).

P.J.Brunton, S.Ma, M.J.Shipston, A.Wigger (1), I.D.Neumann (1), A.J.Douglas, J.A.Russell
Department of Biomedical Sciences, University of Edinburgh. (1) Max Planck Institute for Psychiatry, Munich.

ACTH and corticosterone secretory responses to stressors are attenuated in late pregnant rats (1). Anterior pituitary corticotroph responses to CRH are reduced in late pregnancy, yet ACTH content of dissociated cells is not decreased. However, measured by semi-quantitative in situ hybridisation (ISH), POMC mRNA content in the anterior pituitary decreases to the end of pregnancy, compared with virgin rats. This may follow reduced basal drive from the hypothalamus as expression of CRH and vasopressin mRNAs in the pPVN decreases in pregnancy (2). We have sought reduced central drive during stress by ISH measurement of expression of NGFI-B mRNA in the hippocampus and pPVN, and vasopressin heteronuclear RNA in the pPVN, 30 and 60 min, respectively, after the start of 30 min restraint in a perspex cylinder; rats were killed by decapitation. Restraint increased trunk blood plasma ACTH concentration significantly less in the pregnant rats. NGFI-B mRNA expression in hippocampal sub-fields and the pPVN was significantly less in stressed pregnant rats than in stressed virgins, as determined by silver grain density measurements on ISH autoradiographs. Similarly stimulation of vasopressin heteronuclear RNA expression in the pPVN was reduced in pregnancy. Thus reduced activation of hippocampal and pPVN neurones by restraint stress in late pregnancy may underlie the reduced ACTH secretion. Such reduced responsiveness of the maternal hypothalamo-pituitary-adrenal axis in pregnancy may protect the fetuses from adverse programming by excessive glucocorticoid exposure.

Support: British Council/DAAD;. PJB-Faculty of Medicine Research Student; SM - ORS award.

(1) Neumann I.D. *et al.* (1998) J.Physiol. 508: 289-300.
(2) Johnstone H.J. *et al.* (2000) J. Neuroendocrinol. (In Press).

184.18 *p213*

EFFECT OF GALANIN ON THE ACTIVITY OF THE PROOPIOMELANOCORTIN (POMC) SYSTEM OF THE RAT MEDIOBASAL HYPOTHALAMUS

S. Bouret (1), D. Croix (1), M. Mariot (2), S. Jégou (3), H. Vaudry (3), J. C. Beauvillain (1), V. Mitchell (1)
(1) INSERM Unit 422, IFR 22, 1 Place de Verdun, 59045 Lille Cedex France, (2) Hop Swynghedauw, Lille France, (3) INSERM Unit 413, IFRMP 23, Mt St Aignan France

It has become apparent that galanin as well as POMC-derived peptides such as β-endorphin, play an important role in the hypothalamic circuitry that regulates endocrine functions and appetite behavior. We have recently shown that GalR1 and GalR2 galanin receptor mRNAs are expressed in POMC neurons of the arcuate nucleus, suggesting a direct modulatory action of galanin on the POMC neuronal system. However, the precise role of galanin on the activity of POMC neurons remains to be determined. To elucidate this question, we examined the effects of application of 500 nM galanin on the expression of POMC mRNA and on release of β-endorphin from rat mediobasal hypothalamic (MBH) fragments using an approach previously described (Prevot *et al.*, Endocrinology, 1999). The experiments were performed on tissues maintained in an oxygenated survival milieu. Using an in situ hybridization technique, we show that exposure of MBH fragments to galanin increases significantly POMC mRNA levels in the arcuate nucleus. This increase occurs after at least 60 minutes of application of galanin. Concurrently, in comparison to controls, this application leads to a decrease of β-endorphin levels in the survival medium. The effects of galanin on POMC mRNA expression as well as on β-endorphin secretion in the survival milieu are both antagonized by galantide.

In conclusion, our data show that application of galanin on rat MBH exerts a stimulatory effect on POMC mRNA expression and an inhibitory effect on β-endorphin release into the survival medium. This might implicate a differential role for galanin on synthesis and release of POMC-derived peptides. However, additional experiments are still necessary to understand more precisely these results.

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184.19 *p214*

CHRONIC, PRENATAL STRESS INDUCES POSTNATAL DEPRESSION IN RAT DAMS: REVERSAL WITH NALTREXONE.

J.W. Smythe, J.W. Smith, J.R. Seckl (1), B. Costall
Pharmacy, University of Bradford, Bradford (1) Molecular Medicine Centre, University of Edinburgh, Edinburgh UK

Prenatal stress (PS) produces behavioural impairments in the offspring, which persist into adulthood. The mechanism via which PS alters the progeny involves hormonal fluxes in the mother and fetus; these alter genomic activity. Meaney's group (1) reported that maternal behaviour alone was sufficient to induce genomic changes in stress sensitivity in rat progeny. We investigated whether or not PS might produce behavioural changes in rat dams, which may contribute to effects in the progeny. We chose to use the Porsolt test, which measures immobility in a forced-swim task, and models depression. There are similarities between behavioural effects induced by PS, and those following prenatal exposure to opiates. Therefore, we studied the effects of opiate antagonism on the dams' responses. Pregnant rat underwent daily restraint stress (1h/day, days 10-20 of gestation), or were left undisturbed (control). Half of the control and half of the stressed rats were administered vehicle, while the remainder received naltrexone (NAL;10mg/kg, ip), daily. On post-parturition day 3, dams were placed into a swim tank, and time spent immobile was measured during a 10 min trial. All procedures were approved under Home Office license No. 40/1974. PS significantly elevated immobility scores to approximately 130% of control values. This response was abolished by NAL treatment, which by itself was without effect. These data show that PS can alter behaviour in mothers, and this might contribute to alterations in the offspring. PS may be an important factor in maternal postnatal depression; endogenous opiate systems appear to play a key role in such effects since blockade with NAL eliminated stress-induced postnatal depression.

(1) Francis *et al.* (1999) Science 286: 1155-1158

184.20 *p215*

EFFECT OF GONADAL STEROIDS REPLACEMENT ON HYPERTONIC SALINE STIMULATED VASOPRESSIN RELEASE IN THE FEMALE RAT.

M.J. Evans, S.A.Chaudhary, M.L.Forsling
Neuroendocrinology Research Group, KCL, Guy's Campus, London, UK.

Basal plasma vasopressin (AVP) levels alter over the oestrous cycle and reflect progesterone (P) and oestrogen (E) concentrations. The AVP response to raised plasma sodium levels is reduced in ovariectomized rats. This study was carried out to determine the effect of ovarian hormone replacement on stimulated AVP release. Procedures were conducted in female Sprague-Dawley rats weighing approx. 300g in accordance with the UK Animals (Scientific Procedures) Act 1986. Animals were ovariectomized under anaesthetic and given P (n=8) only or P+E (n=10) as subcutaneous silicone implants in concentrations to mimic those of basal levels present on day oestrus. Under anaesthesia, both venous and arterial cannulae were inserted 5 days later. All studies were conducted between the hours of 11:00 and 16:00 after 48 hours recovery from surgery. A 30-minute control period of 0.15M NaCl infusion at 50 μl/min was followed by 1.5M NaCl infusion for a 90 min period and blood samples taken at time points 0, 45 and 90 after the start of hypertonic infusion. Packed cell volume (PCV) was measured, samples centrifuged for 5 mins and plasma collected for sodium and AVP determination. Plasma AVP concentrations were determined by radioimmunoassay. Statistical evaluation by ANOVA and t-test with Tukey-Kramer multiple correction. The mean plasma AVP levels increased in the presence of P+E by 8.14 ± 1.42 μU/ml compared with 3.34 ± 0.97 μU/ml in P alone ($P<0.001$). The slope of regression of plasma AVP to sodium concentrations had a larger gradient in the case of P+E than P alone ($P=0.037$). Therefore, addition of oestradiol, in the presence of progesterone replacement, provided an increase in both sensitivity and release of the AVP response to elevated plasma sodium levels.